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Development of the smooth muscle foam cell: Uptake of macrophage lipid inclusions

(cholesteryl ester metabolism/cultured cells/phagocytosis)

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ABSTRACT **A possible mechanism for the formation of smooth muscle foam cells in the atherosclerotic lesion was explored. Cultured macrophages (J774 cell line) were induced to form cytoplasmic cholesteryl ester inclusions by exposure to acetylated low density lipoprotein in the presence of cholesterol-rich phospholipid dispersions. The macrophages were disrupted by brief sonication, and the inclusions were isolated by flotation. When these inclusions were placed in direct contact with cultured smooth muscle cells, cellular uptake of the inclusions in a time- and dose-dependent manner was observed. Light and electron microscopy indicated the presence of lipid inclusions throughout the cytoplasm of the cells. Uptake of inclusion lipid by the smooth muscle cells was inhibited by several metabolic inhibitors, indicating that the process is dependent on metabolic activity. A modest but significant hydrolysis of the cholesteryl ester was observed, showing that the stored cholesteryl esters are metabolically available.**

The accumulation of cholesteryl ester inclusions within cells of the large vessels has been established as one of the early events in the development of atherosclerosis (1–3). This deposition leads to the formation of cholesteryl ester-loaded cells, which have been termed foam cells. It has been proposed that both macrophages and vascular smooth muscle cells might serve as precursors to foam cells, and the origin and mechanism of formation of foam cells has been the subject of intensive investigation (3–10). Numerous studies have demonstrated that macrophages maintained in tissue culture can be induced to accumulate large stores of esterified cholesterol and to take on the appearance of foam cells (11–14). The mechanism underlying this accumulation has been shown to be the cellular uptake of either chemically modified or abnormal serum lipoproteins by receptors on the macrophage that do not downregulate as the cells continue to accumulate sterol (15). Although the macrophage is now an obvious candidate as a precursor to the foam cell, many studies have shown that some of the cholesteryl ester-loaded cells present in atherosclerotic vessels have characteristics of a smooth muscle origin (3–7). To date, no satisfactory mechanism has been proposed by which smooth muscle cells in vessels could accumulate large stores of esterified cholesterol. The unregulated lipoprotein receptors found on macrophages have not been found on smooth muscle cells, and the low density lipoprotein (LDL) receptor present on smooth muscle cells is rapidly downregulated upon an increase in cellular cholesterol content (16). Also, attempts to extensively load cultured smooth muscle cells with cholesteryl ester have been largely unsuccessful (16–21). Thus, the question remains as to how these cells acquire extensive deposits of esterified cholesterol.

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In this report we present a model for a mechanism by which smooth muscle cells could acquire cholesteryl ester. We propose that the cholesteryl ester inclusions found in smooth muscle cells are not synthesized within these cells but rather are cholesteryl ester droplets synthesized in macrophages, liberated by macrophage lysis, and taken up intact by smooth muscle cells. Thus cholesteryl ester inclusions within smooth muscle foam cells would be "recycled" macrophage inclusions. Using cultured smooth muscle cells and lipid inclusions isolated from cultured macrophages, we have obtained experimental results that support this model.

MATERIALS AND METHODS

Analytic and Preparative Procedures. Protein concentrations were determined by a modification of the Lowry procedure (22) using bovine serum albumin (BSA) as the standard. Lipids were extracted by the method of Bligh and Dyer (23), and mass determinations of free and esterified cholesterol were made using gas–liquid chromatography (24). Estimations of contents of isotopically labeled free and esterified cholesterol were made using TLC (25). Evaluations of the physical state of lipid inclusions were made using polarizing light microscopy (26). Free cholesterol-rich phospholipid dispersions and apo-high density lipoprotein/phosphatidylcholine acceptors were prepared as described (25). Lipoproteins were prepared from fresh human plasma by the method of Hatch and Lees (27). LDL were acetylated by the method of Goldstein *et al.* (28). Sandoz compound 58–035, an inhibitor of acyl CoA:cholesterol acyl transferase (29), was the gift of John Heider.

Cell Culture. Monkey smooth muscle cells were obtained from Steven J. Adelman, Bowman Gray School of Medicine. Rabbit smooth muscle cell lines were established from explants of the abdominal aorta of 8-month-old New Zealand white rabbits. A normal human skin fibroblast cell line (GM 3468) was obtained from NIGMS Human Genetic Mutant Cell Repository. These cell lines, as well as the Fu5AH rat hepatoma cell line (30), were routinely maintained on minimal essential medium (MEM) supplemented with basal medium Eagle vitamins and 10% (vol/vol) fetal bovine serum. The J774 mouse macrophage cell line (31) was maintained on Williams' Medium E supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum.

Preparation of Lipid Inclusions. Cultures of J774 macrophage cells, which were 75% confluent, were exposed to Williams' Medium E containing acetylated human LDL (25 μg/ml), sonicated free cholesterol-rich phospholipid dispersions (250 μg of free cholesterol per ml; free cholesterol/phospholipid molar ratio, >2), and 1% fatty acid free BSA for

Abbreviations: LDL, low density lipoproteins; BSA, bovine serum albumin.

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3 days. For preparations of inclusions that contained radiolabeled cholesterol, [7-^{3}H]cholesterol (1 μCi/ml; 1 Ci = 37 GBq) was added to the loading medium. This procedure results in the deposition of intracellular lipid inclusions that display optical anisotropy when observed by polarizing light microscopy indicating that the lipids are stored in a liquid–crystalline or crystalline state. Cultures that have isotropic (liquid) inclusions were prepared by exposure of J774 macrophages to the medium described above that also contained oleic acid (150 μg/ml). Cholesteryl ester loading of the Fu5AH hepatoma cell line was carried out as described (25).

Isolated lipid inclusions from either cell line were prepared under sterile conditions as follows: Lipid-loaded cells were harvested by scraping with a sterile rubber policeman. The cells were collected in phosphate-buffered saline (PBS, see ref. 41) and disrupted by sonication in a bath sonicator. Inclusions were separated from cellular debris by flotation at 1000 × *g* for 5 hr. Lipid analysis of the isolated inclusions revealed that greater than 90% of the cholesterol in the inclusions was esterified. Radiolabeled inclusions prepared as described above had a specific activity of 1 μCi/mg of esterified cholesterol. Inclusions were resuspended in MEM supplemented with 10% (vol/vol) calf serum.

Inverted Culture Technique. Cells to be loaded by exposure to isolated inclusions were grown to confluence on glass coverslips (22 × 22 mm). To initiate the loading, the coverslips were inverted over a plastic ring (3 mm thick, with an inner diameter of 25 mm) in a 35-mm Petri plate. In this way the cell monolayer was suspended upside down above the bottom of the dish. Medium containing inclusions was then added to the dish. The inclusions rapidly floated to the top of the medium, coming into direct contact with the cells. At the end of the incubation period, the coverslip was removed and washed twice with PBS containing 0.2% BSA and three times with PBS without BSA. The coverslip could be examined microscopically at this point or could be used for further analysis as follows. Cells were removed from the coverslips by trypsinization and collected by centrifugation. Cell pellets were resuspended in PBS without calcium and magnesium and sonicated briefly in a bath sonicator to disrupt the cells. Aliquots of the sonicate were analyzed for protein and lipid content. This technique for cell preparation ensured that cholesteryl ester inclusions found associated with the cells were resistant to removal by extensive washing, trypsinization, and centrifugation.

Electron Microscopy. Transmission electron microscopy of lipid-loaded smooth muscle cells was performed on cells harvested by trypsinization and collected by centrifugation at 1000 × *g* for 10 min. Cells were fixed with 4% (vol/vol) formaldehyde/1% glutaraldehyde in PBS for 15 min at room temperature, postfixed with 1% OsO_4 in 0.1 M S-collidine buffer (pH 7.4) for 90 min, dehydrated with a graded series of ethanol solutions, and embedded in Epon.

RESULTS

To test the hypothesis that smooth muscle cells can acquire cholesteryl ester inclusions by taking up macrophage inclusions, we obtained cholesteryl ester-rich inclusions from cultures of the J774 mouse macrophage cell line and incubated these lipid inclusions with cultured rabbit aortic smooth muscle cells. Initial attempts to load monolayers of smooth muscle cells with cholesteryl ester by simply adding macrophage lipid inclusions to the culture medium were unsuccessful. The inclusions quickly floated to the surface of the medium suggesting that direct cell-inclusion contact might be required. When gentle agitation of the cultures on a rotary shaker did not significantly improve such contact, we developed an inverted culture technique. In this procedure, smooth muscle cell monolayers were first grown on glass coverslips. Experiments were initiated by removing coverslips from the culture dishes and inverting each coverslip on a plastic ring in a fresh dish, so that the monolayer was suspended upside down above the bottom of the dish. Sufficient medium containing inclusions was added to each dish to allow contact with the cells. The success of the inverted culture technique is shown in Fig. 1. Phase-contrast photomicrographs (Fig. 1 *A* and *B*) of rabbit aortic smooth muscle cells incubated for 3 days with anisotropic inclusions isolated from J774 macrophages show that almost all of the cells in the cultures have incorporated inclusions, and the cytoplasm of many cells is completely filled with lipid droplets. As is illustrated in Fig. 1 *C* and *D*, the inclusions, when viewed with polarizing optics, have the birefringence and cross-formee pattern characteristic of liquid crystals of cholesteryl esters frequently observed in the foam cells present in atherosclerotic lesions (1, 25). An electron micrograph of rabbit smooth muscle cells exposed to inclusions for 24 hr (Fig. 2) shows numerous cytoplasmic inclusions that do not appear to be surrounded by a bilayer membrane. Evidence of the presence of crystalline material similar to the cholesterol crystals found in some foam cells (25) is occasionally seen in inclusion-loaded smooth muscle cells (Fig. 2).

The data in Fig. 3 show that the deposition of lipid by the smooth muscle cells was dependent on both time of exposure to inclusions and concentration of the inclusions supplied to the cells. The concentration of cholesterol recovered in the cells incubated with inclusions for 24 hr reached a level of >600 μg/mg of protein, and ≈90% of the cholesterol was esterified. To establish that the ability to store lipid in response to direct exposure to macrophage lipid inclusions is not unique to this rabbit smooth muscle cell line, a number of different cell lines, including a second line of rabbit smooth muscle cells, monkey aortic smooth muscle cells, and a line of human skin fibroblasts, were exposed to the macrophage inclusions. As shown in Fig. 4, all of the lines of cells were capable of rapid and efficient deposition of cholesterol, indicating that this phenomenon is unique neither to a particular line of smooth muscle cells nor to smooth muscle cells in general. It has been reported that smooth muscle cells rapidly change from a contractile to a synthetic state when maintained in culture (32), a property that might affect the ability of cells to take up exogenous lipid particles. Since all of these cells had been adapted to cell culture for a number

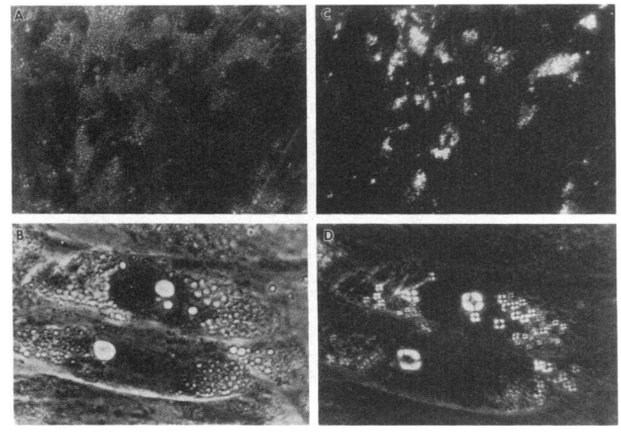

FIG. 1. Photomicrograph of rabbit aortic smooth muscle cells incubated using the inverted culture technique for 48 hr with anisotropic cholesteryl ester inclusions isolated from J774 macrophages. Inclusions were added to culture medium at a concentration of 200 μg of cholesteryl ester per dish. (*A* and *B*) Phase-contrast optics. (*C* and *D*) Same fields, polarizing optics. (*A* and *C*, ×170; *B* and *D*, ×340.)

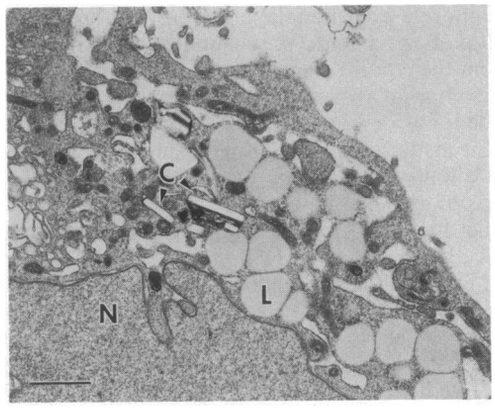

FIG. 2. Transmission electron micrograph of a rabbit smooth muscle cell after 24-hr exposure to lipid inclusions isolated from J774 macrophages. L, lipid inclusion; N, nucleus; C, crystals. (Bar = 0.5 μm.)

of population doublings, we wished to determine whether cells in primary explants of aorta could also take up macrophage inclusions. For these experiments, rabbit aortic explants that had never undergone trypsinization were incubated with inclusions. Microscopic examination of this material revealed numerous lipid-laden cells in the explant outgrowth (data not shown). These lipid-laden cells were present in the confluent, densely populated areas as well as the growing, leading edge of the outgrowth, indicating that subculture is not a prerequisite to inclusion uptake.

Several lines of evidence also suggest that neither the source nor the lipid composition of the inclusions is critical to their uptake by smooth muscle cells. Cholesteryl ester-rich inclusions isolated from Fu5AH rat hepatoma cells were as effective as macrophage inclusions when incorporated into the culture medium at comparable exogenous cholesteryl ester concentrations. The lipid composition of the inclusions also did not affect uptake; anisotropic (cholesteryl ester-rich) and isotropic (triglyceride-rich) inclusions were equally effective. Of particular interest is the observation that when isotropic inclusions were exposed to smooth muscle cells, the lipid droplets initially observed in the cells were isotropic but converted to anisotropic over a period of 2 days (Fig. 5),

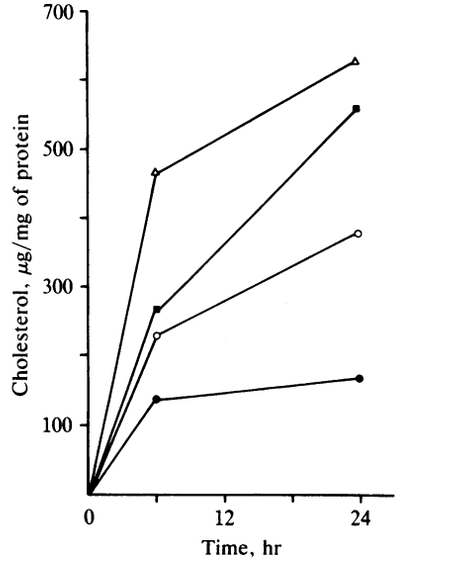

FIG. 3. Accumulation of cholesterol in rabbit aortic smooth muscle cells exposed to various amounts of radiolabeled cholesteryl ester inclusions obtained from J774 macrophages. Macrophage inclusions contained 7% (mol/mol) free [^{3}H]cholesterol and 93% (mol/mol) esterified [^{3}H]cholesterol. Inclusions were provided to confluent smooth muscle cell monolayers in the following amounts (μg of inclusion cholesterol per dish): 50 μg (●), 100 μg (○), 200 μg (■), 300 μg (△). After 24 hr, the coverslips were removed, and the cells were harvested and sonicated. Aliquots of the sonicate were analyzed for protein and total radioactivity. The amount of cholesterol accumulated in 24 hr was calculated on the basis of incorporation of label into the smooth muscle cells. The lipids were extracted, and the relative proportions of free and esterified cholesterol were determined. An average of 90% of the incorporated labeled cholesterol was recovered as cholesteryl ester. Each value plotted represents the average of triplicate coverslips.

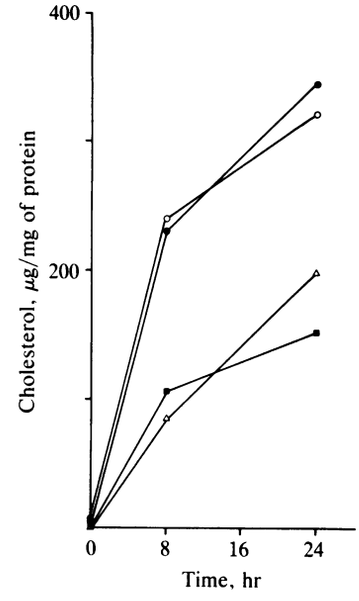

FIG. 4. Accumulation of total cholesterol in two different rabbit aortic smooth muscle cell lines (● and ○), monkey aortic smooth muscle cells (■), and human fibroblast GM3468 cells (△). Radiolabeled inclusions isolated from J774 macrophages were added at 100 μg of cholesterol per dish, and 95% of the inclusion cholesterol was esterified. The mass of cholesterol accumulated was calculated on the basis of incorporation of label into the smooth muscle cells. At the end of the 24-hr incubation period 85–89% of the cellular cholesterol was present in esterified form. Each value plotted represents the average of triplicate coverslips.

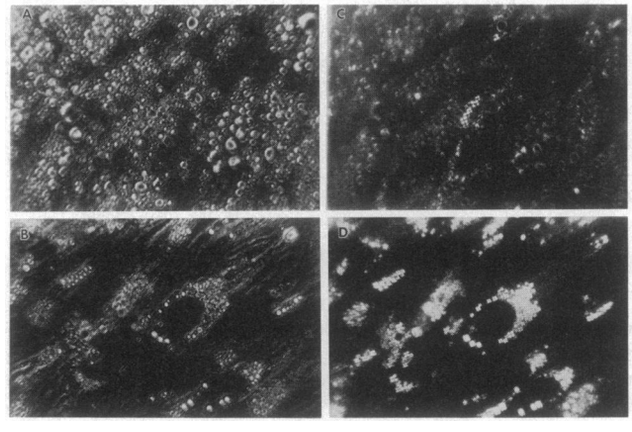

FIG. 5. Change in physical state of lipid inclusions stored in rabbit aortic smooth muscle cells. Monolayers were loaded with isotropic inclusions from J774 macrophages (150 μg of cholesteryl ester per dish) for 24 hr. After washing three times with PBS/0.2% BSA, the coverslips were placed in fresh dishes in MEM with 10% (vol/vol) fetal bovine serum, incubated for an additional 24 or 48 hr, and examined by phase-contrast (*A* and *B*) and polarizing microscopy (*C* and *D*). (*A* and *C*) 24 hr. (*B* and *D*) 48 hr. (×340.)

indicating cellular metabolism of the lipids within the incorporated droplets. Since the liquid state of the inclusions supplied to the smooth muscle cells is a reflection of the triglyceride content of the droplet (33), the liquid to liquid-crystalline conversion of the inclusions when incorporated into the smooth muscle cells suggests that the smooth muscle cells can rapidly, and perhaps selectively, clear the triglyceride codeposited during inclusion uptake.

Experiments were conducted to determine if the incorporated esterified cholesterol was subject to hydrolysis and clearance after the inclusions were incorporated into smooth muscle cells. The loss of [^{3}H]cholesteryl ester from loaded rabbit aortic smooth muscle cells was determined following a 24-hr incubation period in the presence and absence of apolipoprotein–phospholipid complexes that promote the efflux of cellular free cholesterol (25). As shown in Table 1, cultures incubated at 37°C in either medium showed similar but modest decreases in cholesteryl ester content; however, those incubated at 4°C showed no loss of cholesteryl ester. We conclude that there was a small but significant clearance of cholesteryl ester from the loaded smooth muscle cells at 37°C indicating that the stored cholesteryl ester was metabolically available. The free cholesterol generated by hydrolysis was also available for transfer to the plasma membrane and efflux to extracellular acceptors.

To address the mechanism by which smooth muscle cells take up lipid inclusions, the effects of a number of metabolic inhibitors on the uptake process were examined. Little or no inhibition was observed by Sandoz Compound 58-035, a specific inhibitor of acyl CoA:cholesterol acyl transferase, or by 2-deoxyglucose, an inhibitor of glucose transport and glycolysis (Table 2). However, significant reductions of inclusion uptake were achieved by several other compounds that affect cellular energy metabolism (dinitrophenol, carbonyl cyanide *m*-chlorophenyl hydrazone, and NaF), by an inhibitor of protein synthesis (cycloheximide), and by a compound known to interfere with lysosomal function (chloroquine). The dependence of inclusion uptake on active metabolic processes is further supported since the uptake was markedly inhibited when the cells were incubated with the lipid droplets at 4°C for 24 hr. We conclude that inclusion uptake by smooth muscle cells is an active endocytic process.

Table 1. Metabolism of esterified cholesterol by rabbit smooth muscle cells

Treatment	Cholesterol acceptor	% esterified cholesterol	% efflux
Control		80.3 ± 0.5	0
24 hr, 37°C	−	74.8 ± 2.0*	6.5 ± 1.2
24 hr, 37°C	+	74.0 ± 1.2*	21.8 ± 3.8
24 hr, 4°C	−	80.3 ± 1.2	3.0 ± 0.8
24 hr, 4°C	+	79.5 ± 1.2	3.8 ± 1.2

Coverslips containing confluent monolayers of rabbit smooth muscle cells were incubated for 24 hr with [^{3}H]cholesteryl ester inclusions (100 μg of cholesteryl ester per dish). The coverslips were then washed four times with PBS containing 0.2% BSA and were incubated for an additional 24 hr at either 4° or 37°C in MEM containing 0.1% BSA (−) or the same medium containing a free cholesterol acceptor (+) consisting of an egg phosphatidylcholine–high density apolipoprotein complex added at 250 μg of phosphatidylcholine per ml. At the beginning (control) and the end of the 24-hr incubation period, the cells plus medium in each dish were analyzed to determine the total radioactivity in the dish and to quantitate the fraction of the [^{3}H]cholesterol present as cholesteryl ester. At 24 hr, an aliquot of the medium was assayed for [^{3}H]-cholesterol to quantitate the free cholesterol released from the cells over the 24-hr period. The percentage of the total cholesterol (cells plus medium) that is in the medium at 24 hr is presented as % efflux. All values are the average ± SD of four determinations.
*$P < 0.01$.

Table 2. Effect of metabolic inhibitors on the uptake of [^{3}H]cholesteryl ester inclusions by rabbit aortic smooth muscle cells

Treatment	% control
Sandoz 58-035 (1.3 mM)	102.0 ± 21.5
2-Deoxyglucose (10 mM)	94.6 ± 19.4
Sodium azide (10 mM)	70.0 ± 21.3
Cycloheximide (0.35 mM)	58.1 ± 14.1*
Dinitrophenol (1 mM)	54.6 ± 9.6*
Chloroquine (50 uM)	29.7 ± 17.4*
NaF (20 mM)	24.7 ± 17.3*
CCCP (0.25 mM)	22.9 ± 16.6*
4°C incubation	16.5 (n = 3)

Confluent cell monolayers grown on 22 × 22 mm coverslips were incubated for 5 hr with inclusions (as in Table 1) and with each inhibitor at the indicated concentration. Data are the average ± SD of two experiments, each with three coverslips. Results are expressed as % control cultures incubated in the absence of inhibitors. CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.
*$P < 0.01$.

DISCUSSION

The results from this investigation show that cultured smooth muscle cells are capable of storing massive amounts of lipid as intracellular inclusions, thus acquiring properties ascribed to the foam cell in the atherosclerotic lesion. In these experiments, levels of >600 μg of cholesteryl ester per mg of cell protein were achieved, far exceeding published levels for cultured smooth muscle cells (16–21) or, in fact, for macrophages or hepatoma cells (15, 25). The lipid inclusions had several characteristics in common with lipid inclusions in the atherosclerotic lesion, including birefringence and cross-formee when viewed with polarizing light microscopy and the lack of a tripartite membrane when examined by electron microscopy.

Although it has long been appreciated that some foam cells in the atherosclerotic lesion have a smooth muscle cell origin (3), the mechanism by which smooth muscle cells might become lipid laden has not been satisfactorily explained. In contrast, the mechanism by which the macrophage, the other cell type that acquires a foam cell phenotype in the atherosclerotic lesion, becomes loaded with lipid has been well characterized (15). The juxtaposition of these two cell types in the developing lesion led us to propose that smooth muscle cells might become loaded with lipid by phagocytizing cellular debris, including lipid inclusions, generated by macrophage necrosis in the lesion. Smooth muscle cells, while not "professional" phagocytes as are macrophages, have well-characterized phagocytic capabilities. Studies by Garfield *et al.* (34) showed that aortic smooth muscle cells *in vivo*, in acute preparations of aortic rings, and in culture were able to phagocytize a variety of extracellular materials, including necrotic cells. A study by Blaes *et al.* (35) showed that the phagocytic capacity of cultured rat aortic smooth muscle cells is about two and a half times that of cultured rat skin fibroblasts.

In testing our hypothesis *in vitro*, we used cultured aortic smooth muscle cells and exposed them to lipid inclusions isolated from J774 mouse macrophage cells. A critical feature of the uptake of the macrophage inclusions by the smooth muscle cells is that the inclusions must be in direct contact with the cells, suggesting that the inclusions are taken up by an endocytic mechanism and not as a result of extracellular hydrolysis of the lipid components as has been shown for the deposition of triglyceride by cultured cells in response to

exposure to very low density lipoprotein (36, 37). The uptake of lipid was further shown to be both time and concentration dependent, and the phenomenon itself does not appear to be limited to smooth muscle cells, as fibroblasts were also able to be loaded by this method. There also appears to be no specificity with regard to the source of lipid inclusions as inclusions from hepatoma cells were as effective as those from macrophages. A number of metabolic inhibitors known to inhibit phagocytosis (38) were shown to significantly inhibit the uptake of macrophage inclusions. Interestingly, chloroquine, an inhibitor of lysosomal function that has been used to increase cholesteryl ester deposition in response to LDLs, had an inhibitory effect on cholesteryl ester deposition in this system. This inhibitory effect might be explained if one proposed that all particles ingested must go through lysosomes for hydrolysis prior to re-esterification and that the capacity of the lysosomes to physically accommodate the incoming particles has been saturated. However, lysosomal hydrolysis followed by re-esterification does not seem to be required since inhibition of the intracellular esterification reaction had no effect on uptake or on the fraction of intracellular cholesterol found to be esterified.

The metabolic availability of lipids stored in intracellular inclusions has been well established (39, 40). Two lines of evidence suggest that lipid stored by smooth muscle cells in response to exposure to macrophage inclusions is also metabolically available. In the first instance, isotropic inclusions (isotropic by virtue of their high content of triglyceride) were converted to anisotropic inclusions over a period of 48 hr, suggesting a selective hydrolysis and clearance of triglyceride. Cholesteryl esters are also metabolically available as shown by the disappearance of esterified cholesterol over a 24-hr period: however, the fraction of cholesteryl esters cleared per 24 hr is substantially lower than that observed in experiments using the Fu5AH hepatoma cell line (25). It is interesting to note that the presence of an exogenous acceptor of free cholesterol did not stimulate the clearance of cellular esterified cholesterol, as has been reported in a number of other cultured cell systems (15, 25). These findings suggest that smooth muscle cells may have a limited capacity to clear stored cholesteryl ester.

The process of inclusion recycling leading to the formation of myocyte foam cells within the atherosclerotic vessel is consistent with many studies on the natural progression of the early lesion (3–10). These reports have indicated that one of the earliest events in the development of plaque is the infiltration of monocytes followed by the deposition of lipid within these monocyte/macrophages. Although it is thought that some of these lipid-filled macrophages may exit the vessel, carrying away their load of lipid, many clearly undergo lysis, thus liberating the macrophage inclusions into the extracellular environment. The infiltration of macrophages has been shown to be accompanied by the proliferation of medial smooth muscle cells, and this study shows that proliferating smooth muscle cells in culture are capable of incorporating lipid inclusions and acquiring the characteristics of myocyte foam cells.

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