Metabolism of Atherogenic Lipoproteins by Smooth Muscle Cells of Different Phenotype in Culture

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Smooth muscle cells of the rabbit aorta, when grown in vitro, express three distinguishable forms of phenotype (contractile, reversible synthetic, and irreversible synthetic). We compared the interactions of these three smooth muscle phenotypes with rabbit very low density lipoprotein (VLDL), low density lipoprotein (LDL), and very low density lipoprotein from cholesterol-fed rabbits (R-VLDL). R-VLDL showed saturable, high-affinity binding characteristics with each phenotype predominantly through the B/E receptor. The irreversible synthetic cells displayed the greatest binding capacity and the contractile cells, the least. Binding and degradation of normal VLDL was less than that of R-VLDL and higher than that of LDL. Only the irreversible synthetic cells showed substantial (about threefold) cholesteryl ester formation and cholesterol accumulation, and then only when incubated with R-VLDL. Substantial stainable lipid, shown chemically to include triglyceride, cholesterol and cholesteryl ester, was also observed only when irreversible synthetic cells were exposed to R-VLDL. The high capacity of irreversible synthetic-state, smooth muscle cells to bind and accumulate R-VLDL in contrast to the relative immunity of contractile cells may be relevant to the genesis of atherosclerosis in the rabbit and possibly also in humans.

In humans, an early stage of atherosclerosis is characterized by focal cellular thickenings within existing diffuse intimal thickenings. The cells of the focal thickenings are predominantly smooth muscle cells, but the phenotypic expression of many of these cells differs from that of medial smooth muscle cells. This has led to their description as "modified smooth muscle," "poorly differentiated smooth muscle-like cells," and "fibroblast-like." Morphologically, these cells contain few myofilament bundles and increased amounts of organelles involved with synthesis such as rough endoplasmic reticulum, free ribosomes, and Golgi. They closely resemble smooth muscle cells in development and wound repair. Recent studies have shown a differential response to atherogenic stimuli by smooth muscle cells according to the phenotypic state of the cells. Much of this evidence has come from studies involving primary culture of vascular smooth muscle in which changes in phenotypic expression can resemble the cells of focal thickenings. Cells that have been enzyme-dispersed from the adult pig or rabbit aortic media demonstrate this phenotypic change after 6 to 8 days provided they are seeded at less than $10^6$ cells/ml nutrient medium. In the first few days of primary culture while they are in the "contractile" state closely resembling the cells of the intact aortic media, the cells do not incorporate significant amounts of $^3H$-thymidine into DNA or proliferate in response to normolipemic or hyperlipemic whole-blood serum. Following phenotypic change to the "synthetic" state, the cells undergo logarithmic growth in response to whole blood serum. The cells will return to the contractile state upon confluency if...
they have undergone fewer than five cell doublings and have remained in the synthetic state for less than 3 weeks ("reversible synthetic" state).6 However, cells that have been subcultured several times and have undergone multiple cell doublings appear unable to return to the contractile state and are thus in an "irreversible synthetic" state. These cells eventually exhaust their proliferative capacity and become senescent.

In previous reports, we showed that a change in phenotype from the contractile to the reversible synthetic state by primary cultures of pig and rabbit aortic smooth muscle cells was accompanied by a decrease in their ability to degrade 125I-human low density lipoprotein (LDL).6,8 Morphologically, reversible synthetic-state cells grown in 10% hypercholesterolemic rabbit serum for 4 days accumulated lipid droplets, while contractile-state cells under identical conditions were relatively unaffected. This was reflected in an increased incorporation of 3H-sodium oleate into cholesteryl esters in synthetic-state cells.

Since the major lipoprotein of cholesterol-fed rabbits is a cholesteryl ester-rich, very low density lipoprotein or VLDL,9 we have extended these studies to comparisons of β-VLDL metabolism by the three phenotypes of rabbit arterial smooth muscle cells. The uptake of β-VLDL appears to be mediated through the interaction of apoprotein E (a major protein of β-VLDL) with cellular receptors that specifically recognize β-VLDL or apoprotein E-rich lipoprotein in general.10 The former has been clearly defined in macrophages,11 but not in smooth muscle cells. The second receptor, the B/E receptor, interacts specifically also with LDL and VLDL although some catalytic changes in VLDL structure have reportedly facilitated specific binding.12,13 The capacity of β-VLDL to bind to smooth muscle cells and to induce accumulation of lipid has therefore been compared with that of LDL and of VLDL from normal rabbits.

The cells were seeded at $3 \times 10^6$ cells/ml into 60 mm or 90 mm plastic culture dishes (Sterilin). The plating efficiency was greater than 80%, and a near-confluent cell layer was produced. For the study of "contractile" state cells, the primary cultured cells were taken on Days 3 to 4. To obtain "reversible synthetic" cells, unused cultures from the previous week were subcultured and taken on Days 3 to 4 of the first passage (i.e., after 10 to 11 days in culture). Such cells have undergone no more than two cell doublings. "Irreversible synthetic" cells were in their fourth or fifth passage and had been in culture for a total of 4 to 6 weeks and had undergone six to eight cell doublings. They were used 3 to 4 days after their final subculture.

Rabbit aortic smooth muscle cells have a high requirement for glutamine to proliferate. Thus, to achieve two cell doublings (reversible synthetic) or six to eight cell doublings (irreversible synthetic), the cells were grown in the presence of Medium 199 + 5% fetal calf serum (FCS) + 0.2 g/liter glutamine. To minimize differences in the number of receptors due to proliferation, however, reversible and irreversible cells in their final subculture (i.e., at least 3 days before each experiment) were incubated in Medium 199 + 5% FCS to which no additional glutamine had been added. Cells in primary culture were also seeded in Medium 199 + 5% FCS.

Preparation of Serum and Lipoproteins

Whole-blood serum from normolipemic (150 mg/dl to 200 mg/dl cholesterol) and hyperlipemic (900 mg/dl to 1300 mg/dl cholesterol) rabbits was prepared sterile for cell culture. Lipoprotein-deficient serum (LDS) from normal rabbits was prepared by adjusting the salt density to 1.21 g/ml and by ultracentrifuging for 48 hours at 104,000 g. The infranate was dialyzed for 4 days to the osmolality of plasma.

β-VLDL was obtained from the plasma of rabbits made hypercholesterolemic by a diet supplemented with 2% cholesterol as egg yolk. Plasma was overlaid with an equal volume of 0.15 M NaCl and 0.001 M EDTA (pH 7.4) and ultracentrifuged at 110,000 g, at 14°C for 16 hours in a Beckman 50 Ti rotor. The d < 1.006 g/ml supernatant fraction that contained cholesteryl ester-rich VLDL was washed once at this density. Pevikon block electrophoresis15 of this material showed that more than 90% migrated as β lipoprotein. Normal VLDL was isolated from the plasma of normal fasting rabbits by ultracentrifugation under conditions described for the isolation of β-VLDL. LDL was isolated as a d = 1.025 to 1.055 g/ml density fraction by a 24-hour ultracentrifugation at 104,000 g. Lipoproteins were radiiodinated by using a modification of the iodine monochloride method.16 The ratio of moles of iodine bound per mole of protein was routinely kept at less than 1. The distribution of radioactivity showed minor dissimilarities with β-VLDL and normal VLDL, which reflected

Methods

Materials

Sodium 125I-iodide (IMS 30) and [9,10(n)-3H]-oleic acid (5.7 Ci/mmol) were from the Radiochemical Centre, Amersham. Collagenase 4196 CLS was from Worthington Chemical Company, and elastase E-0127 was from Sigma Chemical Company.

Cell Culture

For each experiment, the thoracic and abdominal aortic media and intima from seven 9-week-old New Zealand white rabbits were dispersed into single cells by incubating in 5 ml of 3 mg/ml collagenase for 1 hour (discarded), then 5 ml of 1 mg/ml elastase for 1.5 hours followed by the addition of 5 ml of 3 mg/ml collagenase for another hour. All of the tissue dispersed in the elastase-collagenase mixture, with a yield of 6–7 × 106 viable cells from seven rabbits.

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the differences in apolipoprotein composition. More than 92% of the radioactivity was trichloracetic acid-precipitable with both preparations. Between 12% and 18% of the radioactivity was extractable with chloroform/methanol (lipid-associated) in the various preparations. About 45% of normal VLDL and 58% of β-VLDL radioactivity were precipitable with isopropanol and also comigrated with apo B on polyacrylamide gels. The main difference was in the amounts of label in apo E: about 20% in β-VLDL and less than 10% in VLDL. Conversely, twice as much label migrated with apo C in VLDL than in β-VLDL.

Lipoprotein Binding and Degradation

Cultures were preincubated for 17 hours at 37° C in 3 ml Medium 199 + 5% LDS. At zero time, this medium was removed and replaced with the given lipoprotein in Medium 199 + 5% LDS to a final volume of 3 ml. Binding studies were carried out for 3 hours at 4° C, and degradation studies were performed for 5 hours at 37° C. Parallel studies were carried out in the presence and absence of tenfold excess unlabeled lipoprotein. Additional incubations of lipoproteins without cells were used to correct for non-cell-mediated binding and degradation. In some experiments, the metabolism of increasing amounts of various radioiodinated lipoproteins was measured, whereas in other studies a constant amount (5 μg/ml) of 125I-β-VLDL was incubated with increasing quantities of other unlabeled lipoproteins. For one study, chloroquine at 25 μM, 50 μM, 75μM, and 100 μM was included in the incubation. Binding of radioiodinated 125I-lipoproteins was assayed by measuring cell-associated radioactivity after the medium had been removed and the cells had been washed twice with 0.2% bovine serum albumin in normal saline and then four times in Dulbecco’s balanced salt solution. The degradation of lipoproteins was calculated from the radioactivity in the noniodide trichloracetic acid-soluble fraction.17

For each lipoprotein concentration, both with cells and with no-cell controls and in the presence or absence of tenfold unlabeled excess lipoprotein, triplicate or quadruplicate dishes were used. To determine the number of cells per dish in the binding studies, six dishes of each cell phenotype were treated as for experimental cells then trypsinized and counted in a ZM coulter counter. For degradation studies, the cells of each experimental dish were counted after the degradation products had been removed.

Incorporation of 3H-Oleate Into Cholesteryl Ester and Triglyceride

[9,10(n)-3H]-oleic acid in hexane was evaporated to dryness and complexed to form sodium oleate in a solution containing 12% bovine albumin in 0.9% NaCl. The final concentration of Na oleate in the experimental media was 0.2 mM. Cells grown in 60 mm dishes were incubated for 17 hours in 3 ml of Medium 199 + 5% LDS. The medium was replaced with 3 ml of fresh medium containing unlabeled lipoprotein and 50 μl of 3H-Na oleate-albumin (500,000 cpm per 50 μl), and the cells were incubated at 37° C for 5 hours. The cells were washed and harvested. The cellular lipids were extracted with chloroform/methanol (2:1 vol/vol) that contained 14C-cholesterol as an internal recovery standard and cholesteryl palmitate as a carrier. The lipids were separated by thin-layer silica gel chromatography (solvent system, hexane/diethyl ether/acetic acid, 150:50:2) and were assayed for radioactivity. In each experiment, the value for 3H-cholesteryl ester and 3H-triglyceride represented the mean of duplicate incubations that used quadruplicate dishes for each assay.

Measurement of Cholesterol and Triglyceride Mass

Cells were preincubated for 17 hours in 5% LDS and then for a further 24 hours with unlabeled lipoprotein (mostly 75 μg protein per ml) in 5% LDS. The cells were washed and the lipids were extracted with chloroform/methanol (2:1) that contained a measured internal standard, 5α-cholestanol. The lipid was dried, saponified at 72° C for 2 hours with ethanolic NaOH, and re-extracted in petroleum ether. The total cholesterol mass was quantified as the trimethylsilyl derivative by gas chromatography. A silica capillary column with OV 101 as the liquid phase was used; standards of cholesterol and 5α-cholestanol gave 1:1 ratios; recovery of cholesterol standards was greater than 95%.

For measurements of cholesteryl esters, the chlo-roform/methanol lipid extract was initially separated by thin-layer chromatography using 14C-cholesterol oleate as a recovery standard. The isolated chole-steryl ester was saponified and quantified by gas chromatography as free cholesterol.

Cellular triglyceride was measured enzymatically (Boehringer Mannheim).

Fettrot Staining of Cellular Lipid

Cultures were grown on coverslips and incubated for 17 hours in 5% LDS and then for a further 24 hours with 75 μg/ml of unlabeled lipoprotein in 5% LDS or in 5% LDS alone as control. The cells were fixed in 10% buffered formalin and stained with Fettrot solution, which renders lipid droplets red, and counterstained with Meyer’s Haemalum (modified after Pease16). The degree of lipid accumulation was assessed subjectively by light microscopy.

Results

Cell Size and Protein

With phase-contrast microscopy, cells in the irre-versible synthetic state appear considerably larger than reversible synthetic-state cells, which in turn appear larger than contractile-state cells. This is due
in part to the progressive spreading of the cells on the culture substrate. However, trypsinizing the cells renders them spherical and their volume can be estimated by means of a calibrated 2M Coulter counter giving the values ± standard deviation of 211 ± 32 μm³ (contractile), 380 ± 46 μm³ (reversible synthetic), and 857 ± 68 μm³ (irreversible synthetic). This increase in size is reflected in increases in protein per cell: 0.316 ± 0.079 ng (contractile), 0.595 ± 0.174 ng (reversible synthetic), 1.515 ± 0.369 ng (irreversible synthetic). However, the protein profile of the cell also alters. That is, the cytoplasm of a contractile-state cell contains many myofilaments, while both reversible and irreversible synthetic-state cells contain large amounts of rough endoplasmic reticulum and other synthetic organelles. For these reasons, metabolic measurements have been expressed on the basis of cell number, that is per 10⁸ cells, rather than per protein mass.

**Lipoproteins**

The composition of β-VLDL from hyperlipemic serum and of VLDL and LDL from normolipemic serum is shown in Table 1. (Values are means of two sets of preparations with similar characteristics). The apolipoprotein profiles obtained by polyacrylamide gel electrophoresis on 15% and 3.5% gels are shown in Figure 1. The β-VLDL (90% of which showed beta mobility on Pevikon electrophoresis) contains more cholesteryl ester per protein than normal LDL and less triglyceride per protein than normal VLDL. The β-VLDL is enriched in apolipoprotein E but deficient in apolipoprotein C by comparison with normal VLDL. Unlike normal VLDL, β-VLDL contains substantial apolipoprotein B48 in addition to B100, reflecting a mixture of chylomicron remnants and liver-derived VLDL.

**Lipoprotein Metabolism**

The binding characteristics of rabbit β-VLDL and of VLDL and LDL from normal rabbits were tested in four separate sets of experiments. The data, measured as cell-associated radioactivity at 4°C and expressed as ng lipoprotein protein bound per 10⁶ cells, showed highly consistent differences between the three cell phenotypes. The results of a typical experiment are shown in Figure 2. The data from the four separate experiments have not been meaned, because the cells from different animals and different batches of lipoproteins gave quantitatively slightly different results. But with all sets of experiments, the same similarities and differences were obtained. The binding of LDL to the three phenotypes was about equally specific (displaceable by excess unlabeled lipoprotein) and nonspecific, suggesting substantial non-receptor-mediated binding of LDL. By contrast, the binding of both normal VLDL and of β-VLDL was predominantly specific and saturable over the concentrations tested (5–50 μg protein/ml medium) (Figure 2). There were clear differences in the capacity (Bmax) of the different phenotypes to bind VLDL and β-VLDL (Table 2): the contractile-state cells showed the lowest binding capacity and the irreversible synthetic-state cells showed the highest capacity for these lipoproteins. The binding capacity for β-VLDL by irreversible synthetic cells was invariably greater than for normal LDL. The binding affinity by all three cell phenotypes was substantially higher for β-VLDL than for normal VLDL. Approximately half-saturation was achieved at about 5 μg/ml β-VLDL with the irreversible synthetic-state cells. The Scatchard plots for the binding of β-VLDL and VLDL to both synthetic phenotypes were linear in all cases, suggesting a single binding site. There was a good fit of experimental points to each line.

**Table 1. Composition of Lipoproteins (% by Weight)**

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Protein (%)</th>
<th>CE (%)</th>
<th>TG (%)</th>
<th>FC (%)</th>
<th>PL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-VLDL</td>
<td>7.4</td>
<td>46.3</td>
<td>11.6</td>
<td>8.1</td>
<td>26.6</td>
</tr>
<tr>
<td>VLDL</td>
<td>12.5</td>
<td>4.3</td>
<td>55.8</td>
<td>6.3</td>
<td>21.1</td>
</tr>
<tr>
<td>LDL</td>
<td>23.8</td>
<td>40.7</td>
<td>8.4</td>
<td>3.8</td>
<td>23.2</td>
</tr>
</tbody>
</table>

Values are means of two samples. CE = cholesteryl ester; TG = triglyceride; FC = free cholesterol; PL = phospholipid.
Degradation characteristics of the lipoproteins resembled those for binding with the exception of LDL (Figure 3). Degradation of normal VLDL and of β-VLDL was highly saturable and showed high-affinity, high-capacity characteristics with the irreversible synthetic cells. The capacity of contractile state cells to degrade these lipoproteins was small and that of the reversible synthetic-state cells was intermediate. The degradation of 13-VLDL was greater than of normal VLDL in both synthetic phenotypes. However, the degradation of LDL appeared to be at least as great with contractile-state cells (which we had observed previously in incubations with human LDL9).

The capacity of the various lipoproteins to interact at common binding sites was examined by defining the degree of displacement of 125I-β-VLDL (5 μg/ml)
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Figure 4. A. Degradation of $^{125}$I-labeled B-VLDL (5 μg/ml) in the presence of increasing concentrations of unlabeled B-VLDL, normal VLDL, or LDL in irreversible synthetic state cells. B. Displacement of $^{125}$I-labeled B-VLDL (5 μg/ml) by unlabeled B-VLDL in irreversible synthetic cells in which B/E receptor activity was suppressed (S) or unsuppressed (U).

by unlabeled B-VLDL, normal VLDL, and LDL from irreversible synthetic state cells (Figure 4 A). Degradation was half-suppressed at concentrations of 5 μg/ml of B-VLDL and of 10 μg/ml of normal VLDL. Initial suppression by LDL also occurred at about 5 μg protein/ml, but only about 60% of B-VLDL degradation was suppressible with excess LDL, in contrast to almost complete suppression with normal VLDL and B-VLDL.

These findings suggested that at least some of the interaction between B-VLDL and the smooth muscle cells occurred through the B/E (LDL) receptor. A further study was therefore carried out in which the B/E receptor had been repressed by preincubating irreversible synthetic cells with 20% FCS for 17 hours. Then a saturating concentration of rabbit LDL (75 μg/ml) was added to the medium +5% LDS. During this latter part of the experiment, $^{125}$I-B-VLDL (5 μg/ml) and increasing amounts of unlabeled B-VLDL were added during a 2-hour incubation at 37°C. As shown in Figure 4 B, the unlabeled B-VLDL reduced the uptake (cell-associated radioactivity) of labeled lipoprotein very much less in the cells in which B/E receptor activity had been suppressed than in cells that had been preincubated in LDS and not exposed to excess LDL.

Degradation of lipoproteins was not mediated through nonspecific surface proteases, since minimal degradation occurred in the absence of cells even in the presence of medium that had been previously incubated with cells. On the other hand, degradation was inhibited by chloroquine, with almost total suppression at a concentration of 100 μM (data not shown). This was associated with marked accumulation of cell associated lipoprotein radioactivity.

**Cholesterol and Triglyceride Accumulation**

The mass of total cholesterol in cells of the three phenotypes following 24-hour incubations with 5% LDS alone or with 5% LDS plus LDL, normal VLDL, or B-VLDL (at 75 μg protein/ml) are shown in Table 3. The larger cell size and the greater content of membranous organelles were reflected in the greater cholesterol mass in the synthetic, compared with the contractile, phenotype (see the lipoprotein-free incubations). The addition of 120 μg/ml cholesterol of LDL failed to increase the cellular cholesterol over that in lipoprotein-free incubations. Incubations with 400 μg/ml cholesterol of B-VLDL doubled the cholesterol mass of the reversible-synthetic cells and more than tripled that in the irreversible-synthetic cells. The amount of triglyceride rose in parallel with cholesterol, with the greatest levels in irreversible-synthetic state cells incubated with B-VLDL (56 ± 2 μg/10⁶ cells) compared with 28 ± 5 μg/10⁶ cells in lipoprotein-free incubations.

In further experiments, the incubations contained amounts of LDL or B-VLDL that were equivalent in terms of cholesterol content. However, tripling the LDL cholesterol concentration in the medium failed to raise the cholesterol mass in the irreversible-synthetic cells beyond 21 μg/10⁶ cells, whereas B-VLDL induced a three- to fourfold increase. Whereas cholesteryl ester contributed only about 5% to the cholesterol mass in irreversible-synthetic cells incubated without lipoprotein, this rose to 30% of the total following exposure to B-VLDL. The rise in the total

<table>
<thead>
<tr>
<th>Cell phenotype</th>
<th>LDS (μg total cholesterol per 10⁶ cells)</th>
<th>LDL</th>
<th>N-VLDL</th>
<th>B-VLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contractile</td>
<td>6 ± 1</td>
<td>8 ± 1</td>
<td>ND</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>Reversible synthetic</td>
<td>14 ± 4</td>
<td>24 ± 6</td>
<td>ND</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>Irreversible synthetic</td>
<td>22 ± 2</td>
<td>23 ± 3</td>
<td>28 ± 4</td>
<td>74 ± 8</td>
</tr>
</tbody>
</table>

Each value represents the mean ± standard deviation of between three and five separate determinations. ND = not determined; LDS = lipoprotein-deficient serum.
cholesterol content of the cells was therefore due to both free cholesterol and esterified cholesterol, although the percent increment was much greater for the latter.

**Incorporation of Na Oleate into Cell Lipids**

The incorporation of $^3$H-Na oleate into cellular cholesteryl ester was influenced by lipoproteins and cell phenotype, and the changes resembled those observed for cholesterol mass. The means for three experiments are shown in Table 4. The baseline esterification rates with LDS were twice as great with the two synthetic-state cells as with the contractile-state cells. Incubations with LDL, VLDL, or $\beta$-VLDL led to minimal changes with the contractile cells. Whereas neither LDL nor VLDL stimulated incorporation of $^3$H-oleate into cholesteryl esters in incubations with either form of synthetic cell, incorporation rose by about 50% in the presence of $\beta$-VLDL. The findings for $^3$H-oleate incorporation into cellular triglyceride were almost identical; the only clear changes related to an approximate 50% increase when $\beta$-VLDL was incubated with reversible and irreversible synthetic-state cells (data not shown). It should be noted that the triglyceride content of $\beta$-VLDL was much less than that of VLDL, yet the latter failed to stimulate oleate incorporation into triglyceride above that seen with LDS alone.

**Table 4. Incorporation of $^3$H-Sodium Oleate Into Cellular Cholesteryl Ester**

<table>
<thead>
<tr>
<th>Cell phenotype</th>
<th>LDS (nmole oleate per $10^6$ cells)</th>
<th>LDL (nmole oleate per $10^6$ cells)</th>
<th>N-VLDL (nmole oleate per $10^6$ cells)</th>
<th>$\beta$-VLDL (nmole oleate per $10^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contractile</td>
<td>2.0 ± 0.2</td>
<td>2.2 ± 0.4</td>
<td>2.0 ± 0.1</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Reversible synthetic</td>
<td>4.1 ± 0.6</td>
<td>4.3 ± 0.4</td>
<td>4.1 ± 0.4</td>
<td>6.0 ± 0.7*†</td>
</tr>
<tr>
<td>Irreversible synthetic</td>
<td>4.0 ± 0.1</td>
<td>4.1 ± 0.1</td>
<td>4.5 ± 0.5</td>
<td>5.8 ± 0.6*†</td>
</tr>
</tbody>
</table>

Each value represents the mean ± standard deviation of three separate determinations. LDS = lipoprotein-deficient serum.

*Significantly different from the same cell phenotype incubated in LDS by unpaired $t$ test ($p < 0.001$).
†Significantly different from the contractile state cell in $\beta$-VLDL by unpaired $t$ test ($p < 0.001$).

Figure 5. Contractile (A), reversible-synthetic (B), and irreversible-synthetic (C) state cells stained with Fettrot solution for lipid droplets and counterstained with Meyer’s Haemalum following a 17-hour incubation in 5% lipoprotein-deficient serum (LDS), then a 24-hour incubation with 75 $\mu$g/ml $\beta$-VLDL in 5% LDS. × 600.
Morphologic Studies

There was a marked variation in the accumulation of stainable lipid when the three smooth muscle cell phenotypes were incubated with the three classes of lipoprotein. Incubations of all three smooth muscle phenotypes with 5% LDS or with LDL (120 μg/ml cholesterol) led to minimal Fettrot staining. The contractile-state cells also failed to show more than minor lipid droplets when exposed to VLDL or β-VLDL; however, both synthetic phenotypes showed increased lipid staining when incubated with VLDL or with β-VLDL; by far the greatest lipid staining was in irreversible synthetic cells incubated in β-VLDL (Figures 5 and 6).

In the presence of 10% unfractionated hyperlipemic rabbit serum, marked lipid accumulation occurred in the irreversible synthetic cells, which was of the order seen with β-VLDL incubations.

Discussion

The main finding was related to the marked heterogeneity among the three smooth muscle cell phenotypes with respect to lipoprotein metabolism. There were, in addition, significant differences in the metabolism of the three classes of rabbit lipoproteins. Arterial smooth muscle cells, tested early in primary culture while they still retained the morphological features of contractile cells, showed a very low capacity to bind and to degrade VLDL and LDL from normal rabbits and β-VLDL from cholesterol-fed rabbits. This was associated with minimal accumulation of cellular cholesterol, low esterification of cholesterol, and morphological evidence of no increase in lipid.

By contrast, smooth muscle cells that had undergone phenotypic change to the synthetic state in culture had a higher capacity (expressed per unit cell) to metabolize lipoproteins, specifically β-VLDL and normal VLDL. The greatest accumulation of cellular cholesterol, which was reflected in marked lipid staining, occurred when β-VLDL was incubated with cells that were irreversibly in the synthetic state. With all cells, a much higher proportion of VLDL and β-VLDL was bound through a saturable process than was the case for LDL.

Paradoxically, contractile-state cells appeared to degrade at least as much LDL as the synthetic-state cells, which is consistent with our earlier finding of more degradation of human LDL by contractile- than by synthetic-state cells. Van Lenten et al. have also recently reported a sharp reduction in the degradation of human LDL by human monocytes after the first 2 days in culture.

A notable finding was the relative immunity of the contractile-state cells to cholesterol enrichment, even when they were incubated with β-VLDL; this reflects their low capacity to bind the classes of lipoproteins tested. Even the reversible synthetic-state cells, which can revert to the contractile state under appropriate conditions, showed only a modest increase in cholesterol content with LDL and β-VLDL.

Figure 6. Irreversible-synthetic state cells stained with Fettrot solution for lipid droplets and counterstained with Meyer's Haemalum following a 17-hour incubation in 5% LDS, then a 24-hour incubation in (A) 5% LDS alone (control), (B) 5% LDS and 75 μg/ml LDL, (C) 5% LDS and 75 μg/ml normal VLDL, and (D) 5% LDS and 75 μg/ml β-VLDL. × 600.
Yet the reversible synthetic cells did show a high capacity for binding β-VLDL, which was only a little less than that shown by the irreversible synthetic cells; this demonstrated equivalent incorporation of oleate into cholesteryl esters.

In this study we chose to express the data in terms of cell number rather than cell protein. The main reason was that the mass and type of cellular organelles (which may have little bearing on lipoprotein uptake) change markedly with change in phenotype. However, since the size of the cell increases with phenotypic change, the surface area of the synthetic-state cells is larger than that of the contractile cell. If the number of available receptors rises proportionately, then some of our results may be explicable in those terms. That does not, of course, detract from the important consequence that synthetic-state cells are therefore more susceptible to lipid overload. Nor does it negate the significance of the greater uptake of β-VLDL than of the other lipoproteins.

The accumulation of cholesteryl ester by smooth muscle cells so that they resemble the classic “foam cell” has been difficult to demonstrate in culture. This is presumably due to the fact that the LDL or apo B/E receptor, which mediates the uptake of native lipoproteins by smooth muscle cells, is down-regulated as the amount of cellular cholesterol increases. Thus, incubation of subcultured smooth muscle cells in high concentrations of LDL or β-VLDL results in about a twofold increase in cholesterol content. In each of these studies, the percent increase for cholesteryl ester was far greater than for free cholesterol. The accumulation of cholesterol can be increased by using chloroquine and by incubating the cells with chemically modified LDL, which is not subject to regulation by the LDL receptor. Moderate increases in cholesteryl ester in cultured smooth muscle cells have also been achieved with unfractionated hyperlipemic serum. This may be due to the presence in hyperlipemic serum of β-VLDL, the only naturally occurring lipoprotein that can cause a massive (20- to 160-fold) increase in cholesteryl esters in cultured macrophages. Attention has thus focused on β-VLDL as a potentially important atherogenic lipoprotein.

In our studies, β-VLDL increased the cholesterol content of irreversible synthetic cells to a much greater extent than did LDL, at equivalent lipoprotein cholesterol concentrations. This was also true for the incorporation of 3H-oleate into cholesteryl esters; only β-VLDL, not VLDL nor LDL, stimulated oleate incorporation beyond that observed in the absence of lipoprotein. However, other studies have shown that LDL obtained from cholesterol-fed monkeys does stimulate cholesteryl oleate formation.

Irreversible synthetic arterial smooth muscle cells bound β-VLDL by a high-affinity, highly saturable process. Since normal VLDL displaced the binding of β-VLDL almost as effectively as did β-VLDL itself, and since the initial displacement by LDL was equal-
phologically in a synthetic state and are thus different from the contractile-state cells of the media. If the morphologically identical synthetic-state cells in vivo are functionally equivalent to synthetic-state cells in culture, then at least some are likely to be in the irreversible synthetic state due to the large number of divisions that they must have undergone to form the thickening or plaque. Certainly senescent cells (that is, those that have exhausted their proliferative capacity) have been demonstrated in human plaques. The increased susceptibility of these cells (compared to the contractile-state cells of the media) to accumulate lipid in the presence of B-VLDL may contribute to the predilection of diffuse intimal thickenings for atherosclerotic plaque formation.

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