Lipoprotein Uptake and Metabolism by Rat Aortic Smooth Muscle Cells in Tissue Culture

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ABSTRACT

Aortic smooth muscle cells from the rat were successfully grown in tissue culture and shown to have characteristic morphology. \(^{131}I\)-labeled homologous very low density lipoproteins and high density lipoproteins were taken up by these smooth muscle cells during incubation for 48 hours at the stationary phase. Despite multiple washings, a large proportion of the lipoprotein radioactivity associated with the cells was apparently surface bound and trypsin releasable. With both lipoprotein fractions, lipid and protein uptake by the cells measured after trypsinization was related to time and to the amount of lipoprotein protein added to the medium. Compared with protein, there was a disproportionately greater entry of lipid radioactivity into the cells. Light and electron microscope autoradiography localized the label intracellularly over the cell cytoplasm, cell boundaries, and, in some cells, over lysosomes. On the basis of either protein uptake or whole particle uptake, approximately four times as much high density lipoprotein as very low density lipoprotein was taken up by the smooth muscle cells. To assess metabolism and degradation of high density lipoproteins, aortic smooth muscle cells were incubated in fresh unlabeled medium for 48 hours after exposure to \(^{131}I\)-labeled high density lipoproteins. A large proportion of radioactivity released was trichloroacetic acid precipitable, suggesting some release of whole lipoprotein protein; however, these lipoproteins appeared to be modified when they were tested with anti-high density lipoprotein antiserum. Also, water-soluble radioactivity (presumably protein breakdown products) was released in amounts that averaged 3% of the protein label in the cells. These results indicate that although aortic smooth muscle cells growing in tissue culture can rapidly take up lipids and lipoproteins, catabolism of lipoprotein protein is slow. Correlative biochemical and ultrastructural analysis suggests the possibility of regurgitation of noncatabolized lipoprotein protein by reverse endocytosis.

KEY WORDS high density lipoproteins electron microscopy very low density lipoproteins immunoprecipitation atherosclerosis autoradiography trypsinization aging

In previous studies the synthetic (1-5) and the degradative (6-9) pathways of lipid metabolism have been investigated using both whole arteries and cell-free homogenates. These experiments have provided evidence that aortas of various species can utilize different precursors for the synthesis of neutral lipids and phospholipids and that they contain enzymes active in the degradation of complex lipids. However, most investigators agree (9) that the major portion of aortic cholesterol is not derived from synthesis in situ but from the circulation, where it is carried by low and high density lipoproteins.

Recently it has been shown (10, 11) that whole lipoprotein particles can traverse normal aortic endothelium through the plasmalemmal vesicles and introduce lipids into the aortic wall. The estimates made in these studies suggest that such a transport system would be more than sufficient to carry enough lipoprotein particles to account for the accumulation of cholesterol with age. The presence of lipoproteins in the arterial wall has been demonstrated also by immunological and immunohistochemical methods; however, little is known about the metabolic fate of the protein moiety of serum lipoproteins in the arterial wall. The smooth muscle cell which is the predominant...
cell type in the intima and the media of large arteries is also the cell type that proliferates early in the development of atheroma and eventually becomes the lipid-filled foam cell (12). In the present study, to determine the role of the smooth muscle cells in metabolism of serum lipoproteins, we adapted the method of Ross (13) to obtain rat aortic smooth muscle cells growing in pure culture. The metabolic behavior of rat aortic smooth muscle cells toward different classes of homologous lipoproteins was investigated by testing whether the various lipoproteins are taken up to the same extent and determining the fate of such particles following intracellular ingestion.

Methods

TISSUE CULTURE METHODS

Male rats of the Hebrew University strain were kept in constant-temperature rooms and maintained on the pelleted diet Am-Rod 931 (14). Thoracic aortas were obtained from either 3-month-old or 17-month-old rats. The rats were anesthetized with ether, shaved, surgically scrubbed, and draped. Using sterile technique, their thoracic aortas were rapidly removed. Usually aortas from two or three rats from the same litter were pooled. The aortas were immediately placed in dissecting medium (modified Dulbecco-Vogt medium) containing Tris buffer at pH 7.4 and twice the concentration of antibiotics usually contained in the medium but without bicarbonate and serum.

Explant cultures of adventitia from the aortas were prepared according to the method of Ross (13) to provide homogeneous cultures of smooth muscle cells. In addition, explants of adventitia from the aortas were carried through similar procedures in parallel to provide cultures of fibroblasts for control studies of growth and morphology. Under a dissecting microscope, each aorta was grossly cleaned of loosely adherent fat, rinsed, and cut into smaller circular segments in petri dishes (60 mm in diameter) containing small amounts of dissecting medium. These rings of aorta were cut open to expose the intimal surface, and intimal-medial shreds of less than half the aortic wall thickness were stripped from the surface with very fine-tipped forceps and cut into approximately 1-mm squares for explants. The explants were transferred with a small amount of dissecting medium into Falcon tissue culture flasks (75 cm²); a small amount of medium was added to cover the explants, but it was used sparingly to promote adherence of the explants to the plastic surface. The flasks were incubated at 37°C in an atmosphere of 95% O₂-5% CO₂ and were left loosely capped for the first 24 hours to ensure equilibration of the gas phase. After the first few days, small amounts of additional medium were added until the full volume (approximately 10 ml) was reached. Cells were then fed twice weekly by replacing approximately two thirds of the medium in the flask with fresh medium. Pieces of adventitia were minced and treated comparably for preparation of adventitial fibroblasts. Fibroblasts usually began to grow out of adventitial explants from 3-month-old rats in 4-7 days, and smooth muscle cells usually began to grow out of intimal-medial explants between 10 and 14 days. After 2-4 weeks, when growth appeared to be confluent or when the rate of multiplication slowed, the cells were trypsinized (13). A small sample of resuspended cells was counted in a hemocytometer. Frequently explants were not loosened by the trypsinization procedure but remained adherent; in that case additional fresh medium was added to the original flask and further outgrowth from the explants was continued. In the first passage, cells were transferred to a new flask without subdivision. Subsequent trypsinizations were performed when growth reached confluency, and cells were then subdivided 1:2 or more. Approximately 6.5 × 10⁶ cells were used to seed one 75-cm² flask.

To freeze cells for subsequent use after the trypsinization procedure, the cells were resuspended in medium containing 10% dimethylsulfoxide in a concentration of 2 million cells/ml and frozen in small samples by gradually reducing the temperature from 4°C to -20°C and then to -70°C. For reuse, the frozen cells were thawed in a 37°C water bath, and the cell suspension was transferred to a Falcon flask containing 10 ml of fresh medium. The cells were kept undisturbed for at least 1 week to ensure recovery.

All experiments were performed in plastic Falcon petri dishes (60 mm in diameter) into which 7 × 10⁶ cells had been seeded and had grown to confluency in 4 ml of medium changed every other day. No cells were used for experimentation before the second or after the sixth trypsinization.

PREPARATION OF LIPOPROTEIN FRACTIONS AND IODINATION

Very low density and high density lipoproteins were isolated from rat serum according to the procedure of Havel et al. (15) with an ultracentrifuge (Spinco model L-2 65B). Low density lipoproteins represent only a minor component of rat serum lipoproteins, and they were not isolated. Blood was obtained from rats that had received drinking water supplemented with 10% sucrose for 3 days. Very low density lipoproteins were separated by centrifugation of plasma containing 0.1% ethylenediaminetetraacetic acid (EDTA) for 16 hours at 123,000 g using a 50.1 rotor. The very low density lipoproteins were resuspended in 0.15M NaCl containing 0.01% EDTA and concentrated by additional ultracentrifugation for 16-24 hours at 160,000 g. Following removal of the very low density lipoproteins, the infranatant solution was adjusted to a density of 1.085 with solid KBr and centrifuged for 24 hours at 165,000 g to remove any low density lipoproteins. High density lipoproteins were floated at a density of 1.21 for 48 hours at 165,000 g and further purified by recentrifugation at the same density for 48 hours. Lipoproteins were checked for purity by immunodiffusion. Iodination was carried out with iodine monochloride using the procedure of McFarlane as modified by Bilheimer et al. (16). ¹²⁵I was obtained from the Radiochemical Centre, Amersham, England. All lipoprotein preparations were dialyzed at 4°C overnight against several changes of 0.15M NaCl containing 0.01% EDTA (pH 7.4) to remove free iodine. The label in high density lipoproteins was 98% and that in very low density lipoproteins was 95% precipitable with trichloroacetic acid (TCA). After extraction with a chloroform-

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methanol solution (2:1, v/v) and purification according to the method of Folch et al. (17), the lipid radioactivity in high density lipoproteins was 12–14% and in very low density lipoproteins it was 30–34%. Lipoprotein preparations stained with 1% potassium phosphotungstate and examined under the electron microscope were similar to those described previously (18).

Before they were added to the medium in the petri dishes containing the growing cells, the iodinated lipoproteins were sterilized in IEC filterfuge tubes containing a 0.45μ Millipore filter by centrifugation at 8,000 g for 20 minutes in a Sorval centrifuge at 4°C.

**EXPERIMENTAL PROCEDURE**

Samples (10–500 μliters) of sterilized radioiodinated lipoprotein solutions were added to the fresh medium (4 ml) in each petri dish and allowed to incubate at 37°C up to 48 hours. At selected time periods the medium was removed, and placed in ice; subsequently, a small sample was taken and diluted with water (1:10). A sample of diluted medium was analyzed for TCA-precipitable and TCA-soluble radioactivity after addition of 10% TCA (for a final concentration of 5%) and centrifugation. The cell layer was washed five times with 2 ml of Versene buffer followed by 2 ml of trypsin-Versene solution. After 10 minutes of incubation at 37°C the cells were dispersed in the petri dish by vigorous pipetting with a Pasteur pipette and transferred to counting tubes. The dish was washed once with 2 ml of medium containing 10% serum, and the medium was added to the cell suspension to inactivate the trypsin. A small sample of suspended cells was counted in a hemocytometer. The cells were centrifuged gently (1,000 g for 5 minutes) to form a pellet, and the trypsin supernatant solution was removed, and placed to each control dish. After incubation for 5 minutes at room temperature, TCA was removed, the killed cell layer was rinsed once with 2 ml of buffer, and fresh unlabeled medium was added as previously described. In some experiments, to elucidate the release of adsorbed radioactivity during the chase, cells were trypsinized after the pulse and replated, usually into slightly larger petri dishes (100 mm in diameter). At the end of the experiments after the removal of the medium, the cells were washed an additional time with buffer, trypsinized, washed again, and analyzed for radioactivity as described earlier in this paper.

To identify high density lipoproteins in cells and medium by immunoprecipitation, rabbit antiserum to rat high density lipoproteins was prepared as described by Rachmilewitz et al. (14). Extraction of labeled high density lipoproteins from the cells was carried out as

**TABLE 1**

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>Medium</th>
<th>Fifth wash</th>
<th>Trypsin</th>
<th>Eighth wash</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>131I-High density lipoproteins</td>
<td>24</td>
<td>7,000,000</td>
<td>2,840</td>
<td>15,600</td>
<td>2,100</td>
</tr>
<tr>
<td>131I-Very low density lipoproteins</td>
<td>24</td>
<td>16,400,000</td>
<td>13,450</td>
<td>24,100</td>
<td>2,740</td>
</tr>
<tr>
<td>Free 131I</td>
<td>24</td>
<td>6,800,000</td>
<td>1,600</td>
<td>3,000</td>
<td>1,200</td>
</tr>
</tbody>
</table>

Radioactivity was measured in samples of medium, buffer washes, trypsin solution, and cells after incubation of cells with 131I-labeled lipoproteins and free iodide (not bound to lipoprotein) as described in Methods.
follows. The cells were homogenized in 15% sodium cholate in 0.15M NaCl, diluted 1:5 with NaCl, centrifuged for 30 minutes at 120,000 g, and dialyzed against several changes of 0.15M NaCl containing 0.1% EDTA to remove sodium cholate (14). Immunoprecipitation was carried out at 37°C for 2 hours and at 4°C for 48 hours. The tubes were centrifuged for 20 minutes at 1,500 g and the precipitate was washed with 0.15M NaCl containing 0.1% antiserum. In each experiment the percent of immunoprecipitable counts of high density lipoproteins which had been added in vitro to either unlabeled medium or cells and subjected to the same experimental procedure as the experimental sample served as 100% for the calculation of immunoprecipitable label.

PREPARATION OF CELLS FOR ELECTRON MICROSCOPY AND AUTOGRAPHY

Fixation was carried out in situ for 20 minutes at room temperature using the paraformaldehyde-glutaraldehyde fixative developed by Karmovsky (20) diluted with culture medium. The cultures were washed in buffered 0.25M sucrose for 15 minutes, postfixed in acetate-veronal-buffered 2% osmium tetroxide for 30 minutes, and dehydrated in graded ethanol (35%, 70%, and 95%, 15 minutes each, and 100% twice at 15 minutes) at room temperature. Propylene oxide was added to the petri dish (21), and, following 2 minutes of agitation, the fixed, dehydrated culture was released from the dish in the form of a film, which was then centrifuged, washed with propylene oxide, infiltrated with Epon, and embedded (22). For light microscopy 0.5-1.0μm thick sections were cut from the Epon blocks and stained with 1% toluidine blue dye in 1% sodium borate. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM300 electron microscope at 60 kv. Autoradiography was carried out according to the method of Caro and Van Tubergen (23). For autoradiography at the light microscope level, 0.5-1.0μm thick sections were placed on glass slides and coated with Ilford K3 research emulsion. Following exposure for 1-4 weeks, the slides were developed with Kodak D19b developer, fixed in Kodak acid fixer, washed, and stained. For electron microscopic autoradiography, sections showing pale gold interference colors were placed on grids and coated with Ilford L4 nuclear research emulsion and, following exposure for 3-5 months, were developed in Kodak Microdol X at 20°C, fixed, rinsed, and stained with lead citrate.

ANALYTICAL PROCEDURE

Protein was determined according to the method of Lowry (24). Cells were extracted with a chloroform-methanol solution (2:1, v/v) and purified according to Folch et al. (17). Phospholipids were determined according to Bartlett (25), cholesterol was determined according to Chiamori and Henry (26), and triglyceride was determined by an automated method of Kessler and Lederer (27). Radioactivity was measured with the Auto gamma scintillation spectrometer (Packard).

MATERIALS

The tissue culture medium used was a modification of Dulbecco-Vogt modified Eagle's medium, obtained in weighed dry powder aliquots (Grand Island Biologicals Company), which contained (per 100 ml) 10% fetal calf serum (inactivated at 56°C), 2 ml of 7.5% sodium bicarbonate, 1 ml of a standard nonessential amino acid solution (13), 1 ml of 35° glucose, 2 ml of a 0.2m stock solution of glutamine, 10,000 units of penicillin, and 10 mg of streptomycin. All additions to the medium were kept frozen at -20°C, except bicarbonate, and added fresh before use. The final medium was incubated for at least 24 hours at 37°C to ensure sterility. For preparation of Tris buffer, a 2M Tris solution was titrated to pH 7.4 with 12N HCl. Concentrated Versene solution (EDTA) was prepared by dissolving 2 g of the disodium salt of EDTA, 80 g of NaCl, 4 g of KCl, and 0.5 g of Phenol red solution (for use as an indicator) in 1 liter of water. Samples of this tenfold concentrated stock solution were diluted with water to provide a 20-μg/100 ml or 0.5 μm solution. To provide working buffer, 1 ml of Tris buffer was added to 100 ml of diluted Versene solution. Trypsin (1:250) (Difco Laboratories) as a 0.03% solution was prepared in Versene buffer. Fetal calf serum was obtained from Wellcome Research Laboratories. All solutions for tissue culture were made up in double glass-distilled water and sterilized by filtration using a Millipore filter with combined 0.22μm, 0.45μm, and prefilters pads (Millipore Corporation).

Results

Cultured rat aortic smooth muscle cells were distinguished from aortic fibroblasts by their growth characteristics and their morphology. Smooth muscle cells generally had a longer lag period before outgrowth from explants and after passage reached a stationary growth phase at about 15 days, which was several days later than fibroblasts. The smooth muscle cells, after reaching confluence, continued to multiply and produced multilayered structures. The ultrastructural characteristics of these cells were similar to those described by Ross (13) for guinea pig aortic cells in culture. All cells contained numerous myofilaments with dense bodies, which filled the cytoplasm not occupied by other organelles. The number of plasmalemmal vesicles varied and the cells contained different amounts of endoplasmic reticulum, lysosomes, and lipid droplets. The extracellular components consisted of basement membrane-like material, microfilaments, and small amorphous globules which resembled elastin (Fig. 1A and B).

Both high density lipoproteins and very low density lipoproteins were taken up by smooth muscle cells during incubation (Fig. 2 and Table 1). The procedure for analysis of radioactivity was designed to minimize contamination of cellular radioactivity with trapped label. After removal of the medium, five washes were effective in removing more than 99% of the radioactivity originally added (Table 1). Subsequent trypsinization revealed that a large proportion of the radioactivity associated...
FIGURE 1

A: Electron micrograph of a section of rat aortic smooth muscle cells cultured for 16 days. On the left the membrane (M) which came off the petri dish together with the cells is seen (20). The cells are filled with myofilaments (f) with dense bodies (d) and have numerous plasmalemmal vesicles (v). In the extracellular space there is some basement membrane-like material and material in microfibrillar and amorphous form. B: Area in which the extracellular material resembles elastin (E). A and B are 31,300x; 1μ = 3.1 cm.

with the cell layer (up to 45%) was trypsin releasable. Additional washing was effective in removing residual radioactivity after trypsinization so that total radioactivity in cells represented cellular uptake (Fig. 2). Of the total radioactivity in the cells, the major fraction was chloroform soluble (Fig. 2) and a much smaller proportion was TCA soluble. The remainder of the radioactivity in the
LIPOPROTEIN UPTAKE BY CULTURED AORTIC CELLS

Total uptake

HOURS

FIGURE 2

Uptake of 125I-labeled lipoproteins by cultured smooth muscle cells during a 48-hour incubation expressed as percent of total radioactivity added to the medium. High density lipoprotein (HDL) addition = 140 µg of protein (left) and very low density lipoprotein (VLDL) addition = 140 µg of protein (right). Total in cells represents radioactivity in cell pellet after trypsinization of cell layer and centrifugation followed by resuspension, washing with buffer, and centrifugation repeated twice. Total uptake represents radioactivity present in the cell layer after the removal of medium containing labeled lipoproteins followed by five washes with buffer. This figure was calculated by adding counts recovered during trypsinization to the total in cells. Lipid represents radioactivity in chloroform phase after extraction of TCA precipitate of cells with a chloroform-methanol solution (2:1, v/v). Protein represents radioactivity derived from total radioactivity in cells by subtraction of lipid-soluble and TCA-soluble counts. Each point represents the mean of results from replicate dishes. Dishes contained 4.5 x 10⁶ cells grown for 15 days after passage.

During the first 6 hours of incubation, cellular uptake of radioactivity was rapid. By 2 hours, 75% of the label in cells incubated with high density lipoproteins was in the lipid fraction, despite the presence of less than 15% labeled lipid in the high density lipoproteins added. A comparable phenomenon was observed with very low density lipoproteins (90% vs. 30%). By 24 hours, protein radioactivity from high density lipoproteins constituted 20% of the label in trypsinized cells. During incubation of the medium containing labeled lipoprotein with cells, deiodination apparently occurred, since the proportion of TCA-soluble radioactivity was tripled in 24 hours. The absolute increase in TCA-soluble radioactivity appeared to be more prominent with incubation of very low density lipoproteins than it was with incubation of high density lipoproteins (Fig. 3). Deiodination occurred also in the absence of cells but appeared to be temperature dependent, since there was little increase in TCA-soluble radioactivity with incubation at 0°C.

The protein uptake by cells was related to the amount of protein added to the medium (Fig. 4). With high density lipoproteins, the relationship appeared to be linear up to 250 µg of lipoprotein protein added; saturability of protein uptake was not achieved at concentrations up to 1250 µg of protein. At all concentrations tested, uptake of high density lipoprotein protein exceeded that of very low density lipoprotein protein.

In four other experiments with high density lipoproteins added to cultures of smooth muscle cells, a mean of 13 ng protein/10⁶ µg lipoprotein protein added per 10⁶ cells was taken up in 24 hours, and 28 ng/10⁶ µg was taken up in 48 hours. With very low density lipoproteins, the uptake was 5 ng/10⁶ µg lipoprotein protein added per 10⁶ cells in 24 hours and 18 ng/10⁶ µg in 48 hours. Analysis of precision of replicate cultures incubated with either high density lipoproteins or very low density lipoproteins for 24 and 48 hours yielded a coefficient of variation of 37% for protein uptake.

AUTORADIOGRAPHY

For autoradiography the muscle cells were labeled for 24 or 48 hours with either 125I-high density lipoproteins or 131I-very low density lipoproteins. The distribution of label over the cells was similar with both high density lipoproteins and very low density lipoproteins and represented pro-
Uptake of protein by aortic smooth muscle cells after a 24-hour incubation with variable amounts of lipoprotein protein. Each point is derived from the mean of two replicate determinations of intracellular protein radioactivity after trypsinization and washing of cells calculated from percent uptake times extracellular protein concentration. These data are approximations since the apoproteins of very low density lipoproteins (VLDL) and high density lipoproteins (HDL) do not iodinate uniformly. Dishes contained approximately $11 \times 10^5$ cells grown for 16-17 days after passage.

METABOLISM OF LABELED HIGH DENSITY LIPOPROTEINS

In studies designed to assess the metabolism and the degradation of high density lipoprotein protein by aortic smooth muscle cells, much of the radioactivity released by cells into unlabeled medium after pulsing for 24 hours with $^{131}$I-high density lipoproteins consisted of TCA-precipitable counts (Table 2). The rate of release of TCA-precipitable radioactivity from cells was very rapid initially and declined exponentially (Fig. 7). After a 2-hour pulse with high density lipoproteins a greater proportion of the radioactivity apparently taken up by cells was released during chase compared with that released after a 24-hour pulse. The ratio of labeled lipid to protein in the cells was similar at the end of the 24-hour pulse and at the end of the chase; TCA-precipitable label in the medium contained both lipid and protein in similar proportions. TCA-precipitable radioactivity also was released into the chase medium from cells that had been trypsinized and replated after the pulse. Therefore, it seems that during the procedure of freeing the cells from the petri dish and replating them, only a portion of the releasable label was removed (Table 2). The release of TCA-precipitable radioactivity from pulsed cells into chase medium was also apparent with cells that had been killed by TCA after incubation with labeled high density lipoproteins, but this release was less pronounced. Despite the large release of TCA-precipitable radioactivity from cells during the 48-hour chase, subsequent trypsinization for recovery and analysis of cells released additional radioactivity. This release was much less pronounced if the cells originally had been trypsinized prior to replating before the chase.

Most of the TCA-soluble radioactivity that was released into unlabeled medium during the chase was apparently free iodine, as determined by the distribution of label between water and chloroform phases after peroxidation. Those counts remaining in the water phase after chloroform extraction, which presumably represented radioactive breakdown products of protein, were a small proportion of the radioactivity released into the medium. The killed cells provided a control for nonspecific release of such radioactivity and also provided a methodologic control for the phase-distribution method. When the release of noniodine, water-soluble radioactivity by live cells was corrected in this manner, approximately 0.8% of the protein label present in cells after the pulse was accounted for in this experiment (Table 2). Cells that had been trypsinized and replated to further minimize contamination with TCA-precipitable counts released a similar proportion of labeled breakdown products.
FIGURE 5

Light microscope autoradiographs of sections of aortic smooth muscle cells cultured for 11 days (A and B) or 20 days (C and D). A: Cells labeled with ^141-I-very low density lipoproteins. B–D: Cells labeled with ^141-I-high density lipoproteins for 48 hours. The cells in D were prepared for autoradiography after trypsinization. In all preparations the autoradiographic reaction is concentrated over the cells. A–D are 1300X; 10μ = 1.3 cm.
Electron microscopic autoradiographs of aortic smooth muscle cells cultured for 20 days and labeled with \(^{32}P\)-high density lipoproteins for 48 hours. The autoradiographic reaction is seen predominantly over the cells (A and B); some grains are seen over the cell boundary and over extracellular material (A). Some of the grains are associated with structures resembling secondary lysosomes (C). A: \(7,700 \times; 1 \mu = 0.8 \text{ cm.} \) B: \(20,000 \times; 1 \mu = 2.0 \text{ cm.} \) C: \(31,000 \times; 1 \mu = 3.1 \text{ cm.} \)

Immunoprecipitation with rabbit antirat high density lipoprotein serum was performed to measure the extent to which radioactivity retained by cells and released into the medium was antigenically recognizable. Although 85% or more of the radioactivity remaining in cells after the chase retained the antigenic characteristics of high density lipoproteins, counts released into the chase...
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TABLE 2
Distribution of Radioactivity (counts/min 100 μg DNA) during a 24-Hour Pulse (High Density Lipoproteins) and a 48-Hour Chase Study Using Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Source of recovered radioactivity</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells</td>
<td>Replated cells*</td>
</tr>
<tr>
<td>Seventh wash after removal of labeled medium</td>
<td>3,620</td>
<td>4,620</td>
</tr>
<tr>
<td>Chase medium: TCA-precipitable fraction</td>
<td>126,740</td>
<td>115,390</td>
</tr>
<tr>
<td>TCA-soluble fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>37,550</td>
<td>31,160</td>
</tr>
<tr>
<td>Remaining in water phase‡</td>
<td>722</td>
<td>966</td>
</tr>
<tr>
<td>Eighth wash</td>
<td>7,970</td>
<td>7,080</td>
</tr>
<tr>
<td>Trypsin after chase</td>
<td>46,460</td>
<td>7,230</td>
</tr>
<tr>
<td>Ninth wash</td>
<td>6,920</td>
<td>3,080</td>
</tr>
<tr>
<td>Cells</td>
<td>192,800</td>
<td>202,000</td>
</tr>
<tr>
<td>Cell protein</td>
<td>44,700</td>
<td>43,846</td>
</tr>
</tbody>
</table>

High density lipoprotein (38.5 × 10⁶ counts/min) was added to medium and incubated with smooth muscle cells. Values are means of replicate cultures.

* Cells were trypsinized and replated after a 24-hour pulse, removal of labeled medium, and seven washes with buffer.

† TCA (5%) was added to cells after a 24-hour pulse, removal of labeled medium, and seven washes with buffer.

‡ Radioactivity which remained in the water phase after extraction of the TCA supernatant fraction with chloroform to remove free iodine.

medium were antigenically recognizable as high density lipoproteins to a much lesser extent (Table 3). Aging in vivo in one experiment did not appear to affect the proportion of immunoprecipitable radioactivity recovered in the samples.

In all of the pulse-chase studies with high density lipoproteins, 0.8–10.0% (mean 3.6%) of the protein label in cells after the 24-hour pulse was recovered as radioactive breakdown products in the water phase after extraction of the TCA supernatant fraction of the medium with chloroform to remove free iodine (Table 4). There was a tendency for this fraction to decrease with increased age of cells in vitro.

Discussion

In the present study the method of Ross (13) was used to obtain pure cultures of medial smooth muscle cells from rat aortas. In most experiments 3-month-old rats were used as donors, and the explants were derived from the thoracic aorta. However, cells were also grown successfully from 17-month-old rats and could be sustained for at least six passages in vitro. The growth characteristics of rat smooth muscle cells were similar to those described previously for guinea pig (13) or monkey cells (29). The rat smooth muscle cells reached the stationary phase 2–3 days later than did the guinea pig cells and grew in multilayers. The fine structure of these cells permitted classification as smooth muscle cells according to the criteria of several investigators (13, 30, 31), such as the presence of abundant filaments with dense bodies, numerous plasmalemmal and basal vesicles, and elaboration of extracellular material that resembles elastin. In cultures grown in a petri dish for 40–50 days, the intracellular matrix and filaments accounted for about half of the thickness of the multilayer so that the whole structure in vitro resembled an adult aorta.

The lipoproteins used in the present study were from donors of the same strain of rats used to establish the cultured cells. In previous in vivo and in vitro experiments, the iodine label, which predominantly tags the protein moiety, has proved to be quite stable; however, in this study deiodination was encountered in the presence of the culture medium, presumably as a result of the action of deiodinases in fetal serum (21). This finding did not interfere with the study of uptake of lipoproteins. However, deiodination was considered in the calculation of the results of lipoprotein degradation.

The lipid radioactivity in the cells was much higher than that in the labeled lipoprotein; this finding indicated that a part of the lipid was taken up in nonlipoprotein form. The ¹²⁵I-label resided in the fatty acid portion of lipids and could have been
Rate of release of TCA-precipitable radioactivity from cells after 2-hour and 24-hour incubations of smooth muscle cells in medium containing $^{14}C$-high density lipoproteins (140 μg high density lipoprotein protein/dish) followed by extensive washing and a further incubation with fresh nonlabeled medium. At the end of 48 hours of incubation the dishes contained approximately $2 \times 10^9$ cells grown for 8–11 days after passage. The radioactivity in the cells was 50,000 counts/min after the 24-hour pulse and 16,000 counts/min after the 2-hour pulse. Rate of release of TCA-precipitable radioactivity is expressed as percent of radioactivity of cells released per hour.

interiorized in the form of an esterified lipid molecule by a process of exchange or in the form of free fatty acid following hydrolysis of the ester bond.

The problems encountered in the study of interaction between proteins and cells have been discussed extensively by Ryser (32, 33) and Steinman and Cohn (21), who have pointed out that a rigorous washing procedure must be used to distinguish between uptake and adsorption. Moreover,

<table>
<thead>
<tr>
<th>Age of donor rat (months)</th>
<th>Age of cells in vitro (days)</th>
<th>TCA precipitable*</th>
<th>A†</th>
<th>B‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8</td>
<td>41</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>42</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>29</td>
<td>7.6</td>
<td>2.2</td>
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<td>17</td>
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<td>17</td>
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<tr>
<td>3</td>
<td>42</td>
<td>32</td>
<td>0.8</td>
<td>1.8</td>
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</tbody>
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* Percent of total uptake after pulse.
† Radioactivity which remained in the water phase after extraction of the TCA supernatant fraction with chloroform to remove free iodine as the percent of protein label in cells after pulse.
‡ Medium obtained after chase of cells treated in usual manner.
§ Medium obtained after chase of trypsinized and replated pulsed cells.
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tion. A finding that is analogous to that of Schmidtke and Unanue (35) for serum albumin but unlike that of Steinman and Cohn (21) for soluble peroxidase is that trypsinization of cells, which was used to release the cells from the culture dish, resulted in the removal of a sizable portion of the label. This result was interpreted as an indication that the label remaining after trypsinization and a subsequent wash was localized inside the cell. The localization of the label was further studied with the help of autoradiography at the light microscope level, which confirmed the assumption that the labeled lipoprotein had been interiorized. This finding was further supported by the presence of the autoradiographic reaction over the cytoplasm of cells which had undergone trypsinization.

Comparison of the uptake of high density lipoproteins with that of very low density lipoproteins by smooth muscle cells cultured simultaneously suggests that these results are open to several interpretations. When the comparison was made on the basis of lipoprotein-protein uptake at similar concentrations of lipoprotein protein in the medium, i.e., 600 μg, about four times more high density lipoprotein protein accumulated in the cells in 24 hours than very low density lipoprotein protein. However, if protein uptake means uptake of entire lipoprotein particles and assuming that the protein content of very low density lipoproteins is about 10% and that of high density lipoproteins is about 50%, then the results indicate that for each microgram of high density lipoproteins slightly more very low density lipoproteins had been taken up (Table 5). If, however, the uptake is expressed in numbers of particles rather than in micrograms of lipoproteins and assuming that the uptake by the aortic smooth muscle cells is proportional to the concentration of particles as shown for high density lipoproteins, then at equal particle concentration (e.g., 120×10^14, Table 5) the uptake of high density lipoproteins would again be about four times higher than that of very low density lipoprotein particles. These calculations are only gross approximations and are valid only if it is assumed that all lipoprotein apoproteins are represented and taken up at the same rate. The present studies do not elucidate the mode of uptake of the two lipoproteins, even though they have demonstrated the intracellular presence of their components. If, however, uptake is preceded by attachment to specific sites, then the consideration of lipoprotein–smooth muscle cell interactions becomes more meaningful in terms of particles rather than in terms of micrograms of lipoproteins.

| TABLE 5  |
|------------------|------------------|
| **Calculation of Lipoprotein Uptake by Cells** | Very low density lipoprotein | High density lipoprotein |
| **Lipoprotein** | **Protein(%)** | **Protein(%)** |
| Molecular wt (×10^6) | 3-128 | 25* |
| Range | 600 | 600 |
| Average | 0.6† |
| Concentration in medium | 6000 | 1200 |
| Protein (μg) | 6000 | 1200 |
| Lipoprotein (μg) | 2.8-120 | |
| Particles (×10^14) | 14 | 120 |
| Lipoprotein uptake by | Lipoprotein uptake by | |
| aortic cells | aortic cells | |
| Protein (μg) | 0.3 | 1.2 |
| Lipoprotein (μg) | 3.4 | 2.5 |
| Particles (×10^14) | 0.8 | 24.6 |
| Lipoprotein particle uptake (with 120×10^14 particles in medium) | 6.8×10^11 | 2.46×10^11 |

* Ref. 37.
† Ref. 36.

The problem of utilization of medium protein by cells in culture was investigated extensively in the older literature summarized by Ryser (32). The very careful study of Eagle and Piez (38) pointed out the pitfalls of the methodology used in such studies in which a small contamination of cell protein with medium protein can give erroneously high results. They concluded that in Hela cells in a stationary phase not more than 3–6% of cell protein is derived from medium protein in 48 hours. This observation is contrary to that of Francis and Winnick (39) who found that chicken heart fibroblasts can derive more than half of their tissue protein from soluble protein added to culture medium. It seems plausible that these discrepancies result not only from differences in methodology but also from differences in the behavior of various cell types in culture. In some of the present experiments (not shown) the uptake of very low density lipoproteins and high density lipoproteins by aortic smooth muscle cells was compared with that by adventitial fibroblasts. The adventitial fibroblasts took up considerably more lipoprotein than did the smooth muscle cells.

The uptake of lipoproteins by aortic smooth muscle cells was proportional to the DNA content of the culture; therefore, one might compare the uptake of these particles per cell with that of other proteins by other cell types. In a study on sarcoma
The fate of the ingested lipoproteins was studied in pulse-chase experiments using high density lipoproteins as substrate. The most striking phenomenon was the release of TCA-precipitable radioactivity into the chase medium. This release of label also occurred following trypsinization after pulse labeling and replating of the cells in a new culture dish. This observation indicated that only a portion of the lipoproteins released into the medium could have been derived from particles adsorbed to the plasma membrane and that the bulk had most probably come from lipoproteins not accessible to trypsin action. A similar phenomenon was described by Schmidtke and Unanue (35) who studied uptake of iodinated albumin by macrophages. They found that macrophages pulsed for 1 hour and chased for 18 hours released almost 80% of the label into the chase medium; about 50% of this released label was TCA precipitable. The remaining cell-bound label was 5–7% membrane bound or trypsin releasable and 6–8% intracellular. In the present study, aortic smooth muscle cells that had been pulsed for 24 hours released about 50% of the label into the chase medium; 75% of this released label was TCA precipitable.

Although TCA-precipitable label in the medium represented both lipid and protein in proportions similar to those found in the cells, only about 30% of the label in the medium was still recognizable by a specific antibody, indicating that some change in immunoreactivity had occurred during the process of endo- and exocytosis. At the same time 85–98% of the label that had been recovered from the cells with the help of cholate was immunoprecipitable. The possibility that some of the lipoprotein protein taken up by the cells had undergone intracellular breakdown was analyzed. In view of the rapid rate of iodination, the TCA-soluble radioactivity in the medium had to be corrected for the presence of free iodine; this correction was achieved by separation of the radioactive label between the water and the chloroform phases after peroxidation. As evidence of catabolism, only that portion of TCA radioactivity in the medium that remained in the water phase after chloroform extraction was considered. This portion presumably represented labeled amino acids or other organic iodinated compounds, which would not be expected to be reutilized under these conditions (41). In a series of experiments with both nonreplated and replated cells, about 3% of ingested high density lipoprotein protein had been catabolized during 48 hours (from calculations of data exemplified in Table 2 and summarized in Table 4). This value was much lower than the values reported for the catabolism of albumin (42), hemoglobin (43), or peroxidase (21) by cultured macrophages in which the half time was about 20–30 hours. Sarcoma 180 cells in culture have also been shown to degrade serum albumin at a rapid rate (33). The low rate of intracellular breakdown of high density lipoprotein protein was also reflected by the ultrastructural localization of the label in the aortic cells. Most of the autoradiographic reaction, although intracellular, was seen over the cytoplasm of the cell, and many grains were in the vicinity of the cell surface. Since cellular lipid radioactivity had been eliminated during tissue processing, the reaction presumably resulted from high density lipoprotein protein radioactivity. Occasionally, autoradiographic reaction was encountered over structures resembling secondary lysosomes. Distribution of the label in the cytoplasm was mentioned also by Unanue and Askonas (44) in macrophages labeled with 125I-hemocyanin. This ultrastructural localization of the labeled lipoproteins is compatible with the possibility of regurgitation of noncatabolized protein by reversed endocytosis. The present findings seem to indicate that aortic smooth muscle cells have a rather limited ability to catabolize ingested lipoproteins but might possess a mechanism for their elimination by regurgitation. This limited catabolic ability might result from the relative paucity of lysosomal enzymes in this type of cell or it might be an attribute of a specific lipoprotein. These findings suggest the interesting possibility that during the "roundtrip" the lipoprotein divests itself of some of its lipid, mainly cholesterol. This process could provide a possible pathway for the progressive accretion of cholesterol in aortic smooth muscle cells that occurs in both humans and other species with age.

Addendum

Since this manuscript was submitted, disproportionate uptake of cholesterol from doubly labeled...
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high density lipoproteins ("H-cholesterol and 115I-apoprotein) by cultured human fibroblasts has been reported by J. M. Bailey and J. Butler (Arch Biochem Biophys 159:580, 1973) in accord with results in the present study. It has also been shown that mouse fibroblasts can take up intact labeled triglyceride bound to serum proteins in the medium without prior hydrolysis (J. M. Bailey et al., J Biol Chem 248:1240, 1973).

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