Very Low Density Lipoproteins Promote Triglyceride Accumulation in Macrophages

Sandra R. Bates, Patricia L. Murphy, Zongchen Feng, Takemichi Kanazawa, and Godfrey S. Getz

Incubation of mouse peritoneal macrophages with very low density lipoproteins (VLDL) from normal rats or rhesus monkeys markedly increased the levels of intracellular triglycerides by 10- to 56-fold and was accompanied by the production of oil red O positive vacuoles. The stimulation of triglyceride accumulation in macrophages was time- and concentration-dependent and was specific for VLDL. Three possible mechanisms for the VLDL-stimulated triglyceride accumulation in macrophages were explored: receptor-mediated uptake, action of lipoprotein lipase, and phagocytosis. Macrophage uptake and degradation of 125I-monkey and rat VLDL demonstrated saturable and nonsaturable components. Uptake of 125I-VLDL could be inhibited by unlabeled normal VLDL, although hyperlipemic VLDL was more effective. HDL did not compete to a significant extent. Heparin released lipoprotein lipase-like activity from peritoneal macrophages. Addition of heparin with VLDL resulted in a greater, more rapid elevation in intracellular triglycerides, which was partially inhibited by albumin. Free fatty acid and Intralipid also produced triglyceride accumulation in macrophages. The data showed that all three of the mechanisms examined could contribute to the metabolism of VLDL by macrophages and cause the production of triglyceride-rich cells with a "foamy" appearance, although the evidence suggested that the action of lipoprotein lipase was probably the most important in this process.

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Lipid deposition in the aorta and other major arteries is an important component of the atherogenic process. The accumulation of lipids within cells of the arterial wall is evident as oil red O positive inclusions which give the cells a foamy appearance. These "foam" cells isolated from the aorta are enriched in cholesterol;1-4 the triglyceride content of such cells has not been reported. The diseased vessel itself is enriched with cholesterol esters with a modest increase in triglycerides.5 The accumulation of cholesterol esters is thought to give the cells of the artery wall their "foamy" appearance although high intracellular triglycerides can also produce "foamy" cells very similar in appearance.6,7 Ultrastructural studies have demonstrated that foam cells have smooth muscle cell and macrophage characteristics.8-11 Recently, foam cells isolated from atherosclerotic lesions induced by diet were found to have several histological and functional properties specific for macrophages.10,11 The lipid metabolism of macrophages has been extensively investigated in the past,12 but recent attention has focused on their cholesterol metabolism. Studies have demonstrated that macrophages have cell surface receptors specific for cholesterol ester—rich, beta-migrating very low density lipoproteins (B-VLDL) isolated from animals with diet-induced hyperlipidemia.13,14 B-VLDL were shown to promote extensive cholesterol ester build-up in macrophages.13,14 In contrast, little is known regarding the interaction of macrophages with triglyceride-rich VLDL from normolipemic animals or the possible effects of this lipoprotein fraction on their triglyceride metabolism.

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metabolism. The synthesis and accumulation of triglyceride by other cell types has been studied in vitro by several laboratories. Human skin fibroblasts, bovine aortic endothelial cells, and rat preadipocyte cultures showed increased triglyceride content when incubated in the presence of normal VLDL, although to different extents. The present study describes the accumulation of triglycerides in mouse peritoneal macrophages stimulated by normal triglyceride-rich VLDL.

Methods

Tissue Culture

Unstimulated peritoneal macrophages were isolated from female Swiss-Webster mice according to the method of Cohn and Benson using sterile phosphate-buffered saline (PBS). The exudate was centrifuged, and the supernatant discarded. When contaminating red blood cells were present, they were lysed by a brief exposure to distilled water diluted immediately with Dulbecco's Modified Eagle's medium (DMEM) (Grand Island Biological Company, Grand Island, New York). After centrifugation, the cells were suspended in DMEM plus 10% heat-inactivated fetal calf serum (FCS) (K.C. Laboratory Supply Company, Indianapolis, Indiana). The cells were plated in 1.5 ml aliquots in Multiwell tissue culture plates (Costar, 12 wells/plate). The amount of cellular protein per well ranged from 50 to 150 μg (approximately 2–5 × 10⁶ cells/ml). After 2 hours of incubation at 37°C in a 5% CO₂ atmosphere, the media containing nonadherent cells were removed, and the attached cells were washed twice with PBS. The cells were then incubated for 1 or 2 days in 1.5 ml of DMEM plus 10% FCS. Finally, the macrophages were analyzed or used for experiments. At the start of an experiment, the cells were washed twice with PBS. One ml of test medium consisting of DMEM plus additions was added per well and the cells were incubated for the appropriate time periods at 37°C. At the end of an experiment, the cells were washed three times with PBS and harvested by exposure to 0.1 N NaOH for 20 minutes at room temperature. This short treatment with NaOH did not hydrolyze cellular triglycerides. The dissolved macrophages were neutralized with concentrated acetic acid and extracted immediately using the method of Bligh and Dyer.

Macrophage-like cell lines J774A.1 (J774) and P388D1, interleukin-1 secretor (P388) were obtained from the Cell Distribution Center of the Salk Institute, San Diego, California, and were maintained in DMEM containing 10% FCS. The cell lines were seeded so that the final protein concentration per well was 0.2–0.5 mg.

Arterial medial smooth muscle cells were obtained from outgrowths of explants from the thoracic aorta of Macaca mulatta monkeys (rhesus) as previously described, grown in Basal Medium of Eagle (BME) with 5% calf serum, and used between the 4th and 6th passages (0.3–1.0 mg protein/flask). Experiments were carried out in 25 cm² flask (Falcon) in a volume of 2.0 ml per flask and terminated as described for macrophages.

Lipoproteins

Normal lipoproteins were isolated from the plasma of male Sprague-Dawley rats (King) fed Purina rat chow or from rhesus monkeys fed monkey chow. Normal VLDL isolated from rats or monkeys had similar chemical compositions. The rat VLDL was 55% triglyceride, 11% protein, 25% phospholipid, 5% total cholesterol, and 4% free fatty acid while the monkey VLDL was 52% triglyceride, 11% protein, 21% phospholipid, 12% total cholesterol, and 5% free fatty acid. The apoprotein content, however, was different. The rat VLDL had both high molecular weight (M₅, 335,000) and lower molecular weight (M₅, 240,000) apoprotein B (apo B-100) and little apoprotein E. Human VLDL was isolated from the plasma of fasting normal healthy volunteers. Hyperlipemic VLDL was obtained from a rhesus monkey fed a Purina monkey chow diet supplemented with 2% cholesterol and 25% coconut oil for 2 years (mean serum total cholesterol value = 950 mg/dl). The hyperlipemic VLDL was beta-migrating and cholesterol ester-rich. The plasma was centrifuged at 25,000 rpm for 20 minutes in a SW 27 rotor to remove chylomicrons. VLDL (d < 1.006), low density lipoproteins (LDL) (d = 1.019–1.050) and high density lipoproteins (HDL) (d = 1.063–1.21) were isolated according to the method of Havel et al. with modifications as described by Scanu and colleagues. Then 10 μM phenylmethylsulfonyl fluoride (PMSF) was added to the plasma before lipoprotein isolation to inhibit serine proteases. All lipoproteins were washed by recentrifugation and checked for purity by agarose electrophoresis. The lipoproteins were dialyzed against PBS and sterilized by filtration through a 0.45 μm Millipore-HA filter (Millipore) before use. Lipoprotein-deficient serum (LDS) was obtained at a density greater than 1.21 from normal monkey plasma, dialyzed, heated at 56°C for 30 minutes and filtered. All lipoproteins were used within 2 weeks of preparation.

Protein was determined according to the method of Lowry and phospholipids according to the procedure of Bartlett. The total cholesterol and triglyceride content of the VLDL was analyzed with a Technicon Autoanalyzer II. Fatty acid-free bovine serum albumin was purchased from Sigma, and Intrafillid was purchased from Catter Laboratory.

Assays

Triglyceride and free fatty acid were quantitated using the method of Marsh and Weinstein as modified by Kritchevsky et al. Briefly, the extracted lipids
were separated on silica gel G glass thin-layer chromatography plates (Fisher Scientific Company, Itasca, Illinois) and developed with petroleum ether/ethyl ether/acetonic acid (75:25:1). The separated lipids were charred with sulfuric acid and were analyzed spectrophotometrically. The internal standard was 4-14C cholesterol (56 Ci/mol, Amersham/Searle) added during the extraction, and the results were corrected for recovery (mean recovery = 80%). Gas-liquid chromatographic techniques were used to determine the free and total cholesterol content of the cells and media in Table 6.23

The esterified cholesterol was calculated by subtraction of the free cholesterol from the total cholesterol values and represents the cholesterol in the cholesterol ester. Sodium olate complexed to bovine serum albumin containing 0.2% albumin and three times with PBS were separated on silica gel G glass thin-layer chromatography plates (Fisher Scientific Company, Itasca, Illinois) and developed with petroleum ether/ethyl ether/acetonic acid (75:25:1). The separated lipids were charred with sulfuric acid and were analyzed spectrophotometrically. The internal standard was 4-14C cholesterol (56 Ci/mol, Amersham/Searle) added during the extraction, and the results were corrected for recovery (mean recovery = 80%). Gas-liquid chromatographic techniques were used to determine the free and total cholesterol content of the cells and media in Table 6.23 The esterified cholesterol was calculated by subtraction of the free cholesterol from the total cholesterol values and represents the cholesterol in the cholesterol ester. Sodium olate complexed to bovine serum albumin was prepared according to the procedure of St. Clair et al.32

The secretion of lipoprotein lipase-like activity by cells was measured by incubating cells in DMEM with or without heparin (10 IU/ml, 71 µg/ml) for 5 hours (J774) or 24 hours (peritoneal macrophages). The media were removed, centrifuged to remove any floating cells, and assayed immediately. Media incubated without cells served as the control. Lipoprotein lipase-like activity was assayed using 1H-triolein according to the method described by Nilsson-Ehle and Schotz.33

Interaction of 125I-VLDL and Macrophages

VLDL were iodinated by the iodine monochloride method described by Karin et al.34 as previously outlined.35 Of the 125I lipoprotein, 99% was TCA-precipitable; less than 20% of the label was in lipids. Determinations of the uptake (binding plus incorporation) and degradation of 125I-VLDL were done as described previously35 using the method of Brown and Goldstein.36 The macrophages were incubated for 5 hours at 37°C in Multiwell tissue culture plates (Costar, 24 wells/plate) with 0.5 ml of media containing 125I-VLDL and 2.5 mg/ml LDS. At the end of the incubation, the media were removed and the macrophages were washed three times with cold PBS containing 0.2% albumin and three times with PBS alone. We used 0.2 N NaOH to harvest the macrophages and took aliquots for protein determination and measurements of 125I-radioactivity (= uptake, binding plus incorporation). The media were analyzed for degraded VLDL as previously described35 using the procedures of Brown and Goldstein.36 The data were corrected for binding or degradation which occurred in the absence of cells.

Results

Mouse peritoneal macrophages incubated in medium with 10% FCS contained very little triglyceride (8.2 ± 4.4 µg triglyceride/mg cell protein). Incubation in medium alone for 48 hours did not change the triglyceride content of the cells (Table 1). After exposure to normal VLDL (300 µg triglyceride/ml) for 48 hours, the cells contained many large luminescent oil red O positive vacuoles which were not visible in the control cells, and as shown in Table 1, the triglyceride content of the macrophages increased approximately 10- to 56-fold. Thus, under similar experimental conditions, the macrophages accumulated as little as 97 µg triglyceride/mg cell protein or as much as 355 µg triglyceride/mg cell protein. Although the quantity of triglyceride varied between preparations of macrophages, within each experiment the duplicates were usually within 10% of each other. The reason for these differences between experiments is not clear, but preliminary results indicate that a portion of this variation may be attributable to the number of cells per unit area since, at low cell numbers per well, triglyceride accumulation was inversely proportional to the amount of cellular protein. The age of the VLDL preparation, the state of the macrophages and other unknown factors probably also contributed to the observed variation. The free fatty acid content of cells incubated in 10% FCS was 21.2 ± 11.7 µg/g cell protein. After 48 hours in medium alone or in medium with added VLDL, the cells contained 16.8 ± 12.7 µg free fatty acid or 33.0 ± 18.0 µg free fatty acid/mg cell protein, respectively, indicating only a minor effect of VLDL on the free fatty acid content of the macrophages.

In contrast to macrophages, monkey arterial smooth muscle cells showed a more limited increment in triglyceride levels upon exposure to VLDL. Control cells incubated in LDS contained 19.6 ± 6.9 µg triglyceride/mg cell protein (n = 6), while cells exposed to VLDL (200 µg triglyceride/ml) for 48 hours had 48.1 ± 11.8 µg triglyceride/mg cell protein (n = 10). The ability of other lipoproteins to augment the intracellular triglyceride content of macrophages was examined. The addition of monkey LDL or HDL to the culture medium elevated the intracellular triglyceride content of macrophages but not to the extent seen with VLDL (Figure 1). Thus, at comparable triglyceride concentrations, other lipoprotein classes were much less effective than VLDL in stimulating triglyceride accumulation in macrophages.

### Table 1. Effect of Normal VLDL on the Triglyceride Content of Mouse Peritoneal Macrophages

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Medium</th>
<th>Medium + VLDL</th>
<th>b/a (fold stimulation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg triglyceride/mg cell protein)</td>
<td>(µg triglyceride/mg cell protein)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12.1</td>
<td>130.0</td>
<td>10.7</td>
</tr>
<tr>
<td>2</td>
<td>4.5</td>
<td>97.4</td>
<td>21.6</td>
</tr>
<tr>
<td>3</td>
<td>6.3</td>
<td>355.4</td>
<td>56.4</td>
</tr>
<tr>
<td>4</td>
<td>14.2</td>
<td>200.7</td>
<td>14.1</td>
</tr>
</tbody>
</table>

Macrophages were incubated for 48 hours in medium alone or with normal rat VLDL (300 µg triglyceride/ml).
Figure 1. Exposure of macrophages to normal monkey VLDL, LDL, or HDL. Isolated LDL or HDL were incubated with mouse peritoneal macrophages for 48 hours and compared to the effect of VLDL. All samples were adjusted to 150 μg triglyceride/ml and contained heparin (10 IU/ml). The results are the means ± SD of triplicate determinations. TG = triglyceride. In this experiment, monkeys were fed a normal chow diet enriched with corn oil (18%) 6 hours before being bled to obtain sufficient quantities of VLDL.

The accumulation of triglycerides in macrophages was both time- and concentration-dependent. The triglyceride content of the macrophages increased linearly over the 48-hour period of exposure to VLDL (Figure 2). As shown in Figure 3, the relationship between the extent of intracellular triglyceride accumulation and the concentration of VLDL in the medium was related to the length of the incubation period.

Figure 2. Accumulation of triglycerides in mouse peritoneal macrophages exposed to normal rat VLDL. Macrophages were incubated with VLDL (200 μg VLDL triglyceride/ml) and harvested at the time periods indicated. The results are means and range of duplicate determinations.

With a 24-hour incubation, there was a slight elevation in the triglyceride content at a VLDL triglyceride concentration of 100 μg/ml, followed by a linear increase in proportion to the change in VLDL levels. After 48 hours, the accumulation of macrophage triglycerides was linear up to approximately 100 μg VLDL triglyceride/ml of medium (20 μg VLDL protein/ml), after which it began to plateau. The nonlinear relationship of cellular triglyceride accumulation to VLDL concentration after 48 hours of incubation is probably an indication of the limited capacity of the macrophages to store triglyceride. At 400 μg VLDL triglyceride/ml of medium, the triglyceride content of macrophages increased from 10 μg/mg cell protein at zero time, to 505 μg/mg protein after 48 hours, representing a 50-fold stimulation (Figure 3 B).

The response of macrophage-like cell lines J774 and P388 to normal VLDL was compared to that of mouse peritoneal macrophages under similar experimental conditions. When incubated in medium alone in the absence of VLDL, the J774 cell line had a low intracellular triglyceride content (14.4 μg triglyceride/mg cell protein) similar to that of peritoneal macrophages, while the P388 cells had a higher initial triglyceride content (34.4 μg triglyceride/mg cell protein) (Figure 4). As seen in Figure 4, both macrophage-like cell lines showed an elevation in intracellular triglyceride content in response to the addition of increasing concentrations of normal VLDL to the medium. Although the final triglyceride content was comparable to that seen in peritoneal macrophages, the change in triglyceride content relative to that found in cells incubated without VLDL was the most marked in the peritoneal macrophages (10- to 20-fold), followed by the J774 (five-fold) and the P388 (two-fold) cell lines. VLDL isolated from humans was also effective in elevating the intracellular triglyceride content of macrophages. As shown in Table 2, a substantial accumulation of triglyceride occurred in J774 and peritoneal macrophages after a 24- or 48-hour exposure to triglyceride-rich normal human VLDL.

To explore whether receptor-mediated uptake of VLDL may play a role in triglyceride accumulation, we examined the interaction of [125I]-labeled normal VLDL with the macrophages. The peritoneal macrophages were incubated at 37°C for 5 hours with the indicated concentration of [125I]-labeled normal monkey VLDL and the uptake (binding plus incorporation) and degradation of the lipoproteins were determined. As shown in Figure 5A, the concentration profile of uptake of normal monkey [125I]-VLDL by macrophages suggested both saturable and nonsaturable components. The amount of VLDL degraded (Figure 5B) was one- to two-fold more than that which was taken up; this also suggested saturable and nonsaturable components. A comparison of the uptake of normal monkey and rat [125I]-VLDL is made in Figure 6 A. When the lipoproteins were present at the same protein concentration in the media, uptake (Figure 6 A) and degradation (not shown) of the
Figure 3. Effect of increasing concentrations of VLDL on macrophage triglyceride content. 

A. Macrophages were incubated with increasing concentrations of VLDL for 24 hours. ○ = Experiment 1, ● = Experiment 2. 

B. Macrophages were incubated with increasing concentrations of VLDL and harvested after 48 hours. The results are typical for two such experiments performed. TG = triglyceride. All experiments contained heparin (10 IU/ml). The results represent the mean and range of duplicate samples.

Figure 4. Comparison of mouse peritoneal macrophages to macrophage-like cell lines. Mouse peritoneal macrophages (Peritoneal) and two macrophage-like cell lines (J774 and P388) were incubated for 48 hours in increasing concentrations of normal rat VLDL. A and B represent two different experiments. The results are means of duplicate determinations.
Figure 5. Uptake (A) and degradation (B) of monkey $^{125}$I-VLDL by mouse peritoneal macrophages. The indicated concentration of monkey $^{125}$I-VLDL was added to peritoneal macrophages for 5 hours at 37°C. The data represent the results of four separate experiments. Most points are the means of duplicate cultures with a few single determinations. Degradation was not measured in all experiments.

Figure 6. Interaction of normal $^{125}$I-VLDL and macrophages. A. Increasing concentrations of normal $^{125}$I-VLDL from rhesus monkeys or rats were added to mouse peritoneal macrophages for 5 hours at 37°C. The data represent $^{125}$I-VLDL bound and incorporated by the cells. B. Competition of unlabeled monkey HDL, normal rat VLDL, and cholesterol ester-rich, beta-migrating hyperlipemic VLDL from monkeys fed a diet of 25% coconut oil and 2% cholesterol with normal rat $^{125}$I-VLDL (10 μg protein/ml) for uptake by macrophages. The control value for the uptake of $^{125}$I-normal rat VLDL was 0.4 μg VLDL protein/mg cell protein. Lipoprotein-deficient serum (2.5 mg/ml) was present in all samples.
Table 2. Effect of Human VLDL on the Triglyceride Accumulation in Macrophages

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions to medium</th>
<th>Triglyceride (µg/mg cell protein)</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>12.4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>VLDL</td>
<td>102.6</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>3.7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>VLDL</td>
<td>55.7</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>0.0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>VLDL</td>
<td>152.7</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>0.0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>VLDL</td>
<td>148.5</td>
<td>3</td>
</tr>
</tbody>
</table>

Experiment 1. J774 cells, 300 µg VLDL triglyceride/ml, 2.5 mg albumin/ml, 48 hours of incubation. Experiments 2 and 3. J774 cells, 200 µg VLDL triglyceride/ml, 24 hours of incubation. Experiment 4. Peritoneal macrophages, 200 µg VLDL triglyceride/ml, 24 hours of incubation. No. = number of determinations.

Table 3. Heparin-Releasable Lipase Activity in Medium From Macrophage Cultures

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Additions to assay</th>
<th>Additions to culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>J774*</td>
<td>Serum</td>
<td>None</td>
</tr>
<tr>
<td>Macrophage†</td>
<td>Serum</td>
<td>Heparin (10 IU/ml)</td>
</tr>
<tr>
<td></td>
<td>Serum + 0.5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>M NaCl</td>
<td>34</td>
</tr>
</tbody>
</table>

Data represent the means of duplicate or triplicate determinations and are expressed as nmol oleic acid released in 60 minutes at 37°C per ml of culture medium. Rat serum heated 56°C for 30 minutes served as the source of apoprotein C-II.

*Medium was added to the J774 macrophages for 5 hours, removed, and assayed for lipase activity for a 60-minute time period.
†Medium was added to peritoneal macrophages for 24 hours, removed and assayed for lipase activity for a 90-minute time period.

VLDL from the two species were virtually identical. The uptake was specific for VLDL in that the addition of excess unlabeled normal VLDL competed with itself for uptake by the macrophages (Figure 6B) and monkey HDL did not compete to a significant extent. However, the cholesterol ester-rich, beta-migrating VLDL isolated from monkeys fed a coconut oil plus cholesterol diet were much more effective than normal VLDL in the inhibition of the uptake of labeled normal rat VLDL. Comparable results were obtained with 125I-monkey VLDL (data not shown).

The possible contribution of lipoprotein lipase to triglyceride accumulation was explored after it was reported that J774, a macrophage-like cell line,37 human monocyte macrophages,38,39 and rabbit alveolar macrophages37,38 secreted lipoprotein lipase. To demonstrate that mouse peritoneal macrophages also produced a lipoprotein lipase-like enzyme, medium with or without heparin was added to J774 cells and mouse peritoneal macrophages, removed and tested for lipase activity. The data in Table 3 provide partial evidence for the presence of heparin-releasable lipase activity in medium from mouse peritoneal macrophages with characteristics comparable to that produced by J774 cells. Furthermore, the hydrolysis of triglyceride to free fatty acid stimulated by medium from peritoneal macrophages was inhibited 93% when 0.5 M NaCl was included in the assay.

As shown in Figure 7, the addition of heparin (10 IU/ml) with VLDL (150 µg triglyceride/ml) resulted in a more pronounced increase of triglyceride in macrophages, culminating in a 1.5-fold greater cellular triglyceride content in macrophages incubated for 48 hours with VLDL in the presence of heparin than with VLDL alone (Figure 7). VLDL from either monkeys or rats was equally effective in promoting triglyceride accumulation, which was similarly stimulated when heparin was present in the medium (Figure 8). As demonstrated in Figure 7, the presence of albumin inhibited the increase in intracellular triglycerides for 6 hours but did not prevent its eventual accumulation. Table 4 summarizes the results of several experiments where macrophages were incubated with...
Table 5. Oleic Acid Stimulation of the Intracellular Triglyceride Content of Macrophages

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Macrophage</th>
<th>Oleate concentration (µg/ml)</th>
<th>Cell triglyceride (µg/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>J774</td>
<td>—</td>
<td>23.0</td>
</tr>
<tr>
<td>2</td>
<td>J774</td>
<td>100</td>
<td>150.4</td>
</tr>
<tr>
<td>3</td>
<td>Peritoneal</td>
<td>100</td>
<td>123.4</td>
</tr>
<tr>
<td>4</td>
<td>Peritoneal</td>
<td>125</td>
<td>203.3</td>
</tr>
</tbody>
</table>

Cells were incubated for 24-hours with oleate complexed to bovine serum albumin at a 6.8 to 1.0 molar ratio.

The triglyceride was no longer present in the medium after a 48-hour incubation, indicating that, theoretically, 100 µg of fatty acid was available to the cells if all of the VLDL triglycerides were hydrolyzed via the action of lipoprotein lipase. The data in Table 5 show that such levels of fatty acids added to the medium were capable of causing triglyceride levels to increase in the macrophages. After a 24-hour incubation, 100 µg/ml of oleate produced a substantial increment in cellular triglycerides in both J774 and peritoneal macrophages.

Changes in the cholesterol content of the macrophages during incubation with VLDL were determined. As shown in Table 6, exposure of J774 macrophages to normal rat VLDL resulted in an increase in intracellular triglycerides but did not elevate the cholesterol ester content of the cells. After 24 hours of incubation, the free cholesterol content of the macrophages increased slightly while the cholesterol ester content was actually reduced from control values in all three experiments. The increase in the cholesterol content of macrophages produced by hyperlipemic monkey VLDL which has been reported elsewhere is confirmed here and used for comparative purposes.

The role of phagocytosis was examined using Intralipid, a triglyceride-phospholipid micelle mixture containing no apoproteins such as apo C-II, which is required as an obligatory factor for lipoprotein lipase. Since incubations were done in the absence of serum, the Intralipid should not be a substrate for lipoprotein lipase, although other lipases may hydrolyze the triglycerides. When incubated with the two macrophage cell lines (Table 7), Intralipid caused a 17-fold stimulation in the triglyceride content of J774 and a threefold increase in that of P388 macrophages. Mouse peritoneal macrophages were also enriched with triglyceride after incubation with Intralipid (data not shown). This was not due to a nonspecific adsorption of Intralipid to the cell surface since an experiment using P388 demonstrated that there was no triglyceride associated with the cells after a 2-hour incubation period.
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Table 6. Triglyceride and Cholesterol Content of J774 Macrophages Exposed to Normolipemic or Hyperlipemic VLDL

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions to medium</th>
<th>Triglyceride (µg lipid/mg cell protein)</th>
<th>Cholesterol (µg lipid/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 None</td>
<td>VLDL (normolipemic)</td>
<td>0</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>VLDL (hyperlipemic)</td>
<td>54.3</td>
<td>9.1</td>
</tr>
<tr>
<td>2 Albumin</td>
<td>VLDL (normolipemic)</td>
<td>10.6</td>
<td>14.1</td>
</tr>
<tr>
<td>3 Albumin</td>
<td>VLDL (normolipemic)</td>
<td>49.4</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>VLDL (normolipemic)</td>
<td>9.0</td>
<td>39.3</td>
</tr>
</tbody>
</table>

J774 cells were exposed to normolipemic rat VLDL (200 µg triglyceride/ml, 12 µg total cholesterol/ml) or hyperlipemic monkey VLDL (31 µg triglyceride/ml, 257 µg total cholesterol/ml) for 24 hours. Albumin = bovine serum albumin (10 mg/ml). No. = number of determinations.

Table 7. Effect of Intralipid on Triglyceride Accumulation in J774 and P388 Macrophages

<table>
<thead>
<tr>
<th>Cells</th>
<th>Additions to medium (µg triglyceride/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>J774</td>
<td>10.5 ± 3.2</td>
</tr>
<tr>
<td>P388</td>
<td>59.9 ± 12.5</td>
</tr>
</tbody>
</table>

Cells were exposed to medium alone or containing Intralipid (300 µg triglyceride/ml) for 48 hours. The results are the mean ± S.D. of four separate cultures and are representative of the six experiments performed.

Discussion

The present study demonstrated that normal VLDL produced a marked increase in the triglyceride content of mouse peritoneal macrophages and certain macrophage-like cell lines. The stimulation of triglyceride accumulation in macrophages was specific for VLDL, since low and high density lipoproteins caused only slight changes. The effect of VLDL was both time- and concentration-dependent. In contrast to macrophages, the intracellular triglyceride content of monkey smooth muscle cells was only moderately affected by VLDL. A slight elevation in cellular triglyceride concentrations has been observed in human skin fibroblasts and bovine aortic endothelial cells after exposure to VLDL at levels comparable to those used in this study, while fibroblasts derived from rat foreskin and lung were not affected by VLDL. If hypertriglyceridemic human serum or very high concentrations of VLDL (800 µg VLDL triglyceride/ml) were used, substantial elevations in intracellular triglycerides could be produced in human skin fibroblasts. VLDL isolated from either triglyceride-rich or normal human serum produced comparable, but limited, changes in triglyceride levels in skin fibroblasts. The most pronounced response to normal VLDL was that seen in preadipo-cytes derived from rat epididymal fat pads, which accumulated intracellular triglyceride levels of 2 mg/mg cell protein under specific conditions.

Three mechanisms which might play a role in the observed accumulation of triglycerides in macrophages were explored in this study. They include uptake of VLDL and its associated triglyceride via specific cell surface receptors; action of lipoprotein lipase on VLDL triglyceride in the media resulting in the uptake of the liberated fatty acids by the cells and their incorporation into cellular triglycerides; and phagocytosis of intact triglyceride-rich VLDL. The results gave suggestive evidence that all three mechanisms could have contributed to the elevation in macrophage triglyceride levels. Further detailed studies are in progress to elucidate the relative importance of each to the process.

The interaction of VLDL with cell surface receptors was examined using 125I-labeled VLDL. Mouse peritoneal macrophages possess few LDL cell surface receptors but do have high affinity receptors specific for both hypertriglyceridemic VLDL and β-VLDL, the beta-migrating cholesterol ester-rich lipoproteins from cholesterol-fed animals. Partial evidence for the specific interaction of normal VLDL with macrophage cell surface receptors is presented in this report. Macrophages appeared to take up and degrade VLDL through both saturable and nonsaturable processes. In addition, preliminary results indicated that increasing concentrations of unlabeled monkey LDL had only a slight effect on the uptake of rat 125I-VLDL. HDL did not interfere with the uptake of VLDL, but β-VLDL produced a marked inhibition of the uptake of normal triglyceride-rich VLDL. Thus, the two types of VLDL particles may have different affinities for the same receptor, or, alternatively, the presence of β-VLDL may affect the uptake of normal VLDL by the macrophages. It is of interest that, although the VLDL from rats and monkeys contain different complements of apoproteins B-100, B-48 and E, they both interact with the macrophage receptors in a similar fashion, raising questions about the
identity of the ligand. Evidence from other laboratories has indicated that many cell types are capable of incorporating either intact VLDL particles or components of VLDL. \(^{125}\)I-labeled VLDL has been shown to be taken up and degraded by smooth muscle cells,\(^{41}\) lymphocytes,\(^{42}\) and fibroblasts.\(^ {15, 43}\) The radioactive triglycerides of labeled VLDL were incorporated and metabolized by endothelial cells,\(^ {7}\) L cells,\(^ {44}\) and Ehrlich ascites cells\(^ {45}\) without significant hydrolysis.

Several lines of evidence indicate that the predominant mechanism through which triglycerides accumulate in macrophages is through the action of lipoprotein lipase. First, various types of macrophages secrete lipoprotein lipase\(^ {37-39}\) and, in the present study, mouse peritoneal macrophages were shown to produce a heparin-releasable lipase that was largely inhibited by 0.5M NaCl.

Second, the addition of heparin with VLDL resulted in a faster, more substantial increase in the intracellular triglyceride content of macrophages than seen with VLDL alone. In studies using preadipocyte cultures, heparin caused the release of lipoprotein lipase into the culture media.\(^ {46, 47}\) The cells then synthesized more lipase, resulting in a net increase in the total quantity of lipase per culture which resulted in a greater triglyceride accumulation in the preadipocytes incubated with VLDL and heparin as compared to those not exposed to heparin.\(^ {48}\) The heparin-stimulated accumulation of triglycerides in macrophages probably operates through mechanisms similar to those described for preadipocytes.

Third, bovine serum albumin, which might act as a sink for the released fatty acids, was able to temporarily inhibit the elevation in triglyceride levels in macrophages.

Fourth, free fatty acids that would be generated from the hydrolysis of VLDL triglycerides by lipoprotein lipase, were themselves able to produce triglyceride accumulation in macrophages.

Fifth, since the cholesterol ester-rich, triglyceride-poor β-VLDL was very effective in blocking the uptake of normal \(^{125}\)I-VLDL, it should have been able to prevent the intracellular triglyceride increase stimulated by normal VLDL, if the latter were operating solely through a receptor-mediated process. However, preliminary experiments indicated that co-incubation of cells with normal VLDL and β-VLDL produced a level of intracellular triglyceride accumulation similar to that which would have been expected in the presence of normal VLDL alone.

Sixth, the VLDL from rats and monkeys contain different amounts of apoproteins B-100, B-48 and E, yet both VLDL produced equivalent levels of intracellular triglycerides in macrophages, suggesting that the triglyceride accumulation might be independent of these apoproteins. Lipoprotein lipase would only require the presence of apoprotein C-II. Lipoxygenase-generated products of VLDL metabolism have manifested enhanced interactions with fibroblasts and smooth muscle cells in culture when compared to the original

Finally, a 24-hour incubation of macrophages with increasing concentrations of VLDL did not show the saturation kinetics for triglyceride accumulation expected of a receptor-mediated process. Thus, we conclude that the lipoprotein lipase produced by macrophages is probably of major importance in their accumulation of intracellular triglyceride.

Gianturco et al.\(^ {7}\) proposed that cell surface receptors specific for hypertriglyceridemic VLDL were responsible both for the accumulation of approximately 400 \(\mu g\) triglyceride/mg cell protein after a 24-hour incubation and for the appearance of oil red O stainable droplets in mouse peritoneal macrophages. While some triglyceride uptake may be receptor-mediated, our results with normal VLDL suggest that the bulk of the triglyceride accumulation in the macrophages occurs through the action of lipoprotein lipase. In the work reported by Gianturco et al., normal VLDL did not produce triglyceride accumulation, while the present studies demonstrated a marked increase in intracellular levels of triglyceride upon incubation of macrophages with VLDL from normal rats, monkeys, and humans. The extent of triglyceride accumulation is sensitive to many experimental conditions that could well account for the differences between our results and those of Gianturco et al.\(^ {7}\)

In the work presented here the lipoproteins were from rats, monkeys, and humans, while the macrophages were from mice. Since the VLDL from all three sources produced similar enrichments in macrophage cellular triglycerides, it appears that species differences probably do not play a major role. However, the effect of mouse VLDL was not examined and the possibility that species differences between lipoproteins and cells may have affected the results cannot be entirely ruled out.

Phagocytosis of the intact VLDL particle may also contribute to triglyceride accumulation in macrophages. The elevation in intracellular triglycerides in the cell lines J774 and P388 observed after their incubation with Intralipid suggests the possibility of the apoprotein-independent uptake of micellar particles which also might occur with a VLDL particle. However, further studies are needed to substantiate this point. Since the activator of lipoprotein lipase, apoprotein C-II, was not added to medium containing Intralipid, it is unlikely that this enzyme was involved in the triglyceride accumulation promoted by the artificial micellar particles. On the other hand, the possibility that the macrophage secretes apo C-II cannot be excluded nor can the mediation of another secreted lipase be ruled out.

Zilversmit\(^ {46}\) has proposed that lipoprotein lipase plays an important contributory role in atherogenesis due to its conversion of VLDL to remnant particles. Such products of VLDL metabolism have manifested
triglyceride-rich lipoprotein particle.\textsuperscript{50-53} Although atherosclerotic plaques contain predominantly cholesterol rather than triglyceride, triglyceride accumulation may be a transient phenomenon in the evolution of the atherosclerotic lesion. The remnants remaining after the hydrolysis of triglycerides within the triglyceride-rich chylomicron and VLDL particles may contribute to the cholesterol of the lesion cells. Such a formulation puts added emphasis on the role of triglyceride-rich lipoproteins. The recent reports\textsuperscript{54, 55} that macrophages, which are important cellular components of the evolving atherosclerotic plaque, produce lipoprotein lipase, add support to the notion that these cells may play an important role in the metabolism of VLDL by the arterial wall. We present evidence that lipoprotein lipase not only contributes to macrophage metabolism of VLDL, but that such interactions result in the production of a triglyceride-rich cell with oil red O positive inclusions and a “foamy” appearance.

\section*{Addendum}

After this manuscript had been submitted for publication, a report appeared concerning the role of lipoprotein lipase in the metabolism of VLDL by macrophages which supported many of our observations.\textsuperscript{54}

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