The β-VLDL receptor pathway of murine P388D$_1$ macrophages

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Abstract Very low density lipoproteins S_f 100–400 (VLDL$_1$) from hypertriglyceridemic (HTG) subjects and chylomicrons cause receptor-mediated lipid engorgement in unstimulated macrophages in vitro via the β-VLDL receptor pathway. We now report that the murine macrophage P388D$_1$ cell line possesses the characteristics of the β-VLDL receptor pathway observed previously in freshly isolated resident murine peritoneal macrophages or human monocyte-macrophages. HTG-VLDL$_1$ isolated from the plasma of subjects with hypertriglyceridemia types 3, 4, and 5 interact with P388D$_1$ macrophages in a high-affinity, curvilinear manner. β-VLDL, HTG-VLDL$_1$, chylomicrons, and thrombin-treated HTG-VLDL$_1$ (which do not bind to the LDL receptor) compete efficiently and similarly for the uptake and degradation of HTG-VLDL$_1$. LDL and acetyl LDL do not compete, indicating that uptake of HTG-VLDL$_1$ is via neither the LDL receptor nor the acetyl LDL receptor. Binding of thrombin-treated HTG-VLDL$_1$ to the β-VLDL receptor indicates that the thrombin-accessible apoE, which is absolutely required for interaction of HTG-VLDL $S_f > 60$ with the LDL receptor, is not required for binding to the β-VLDL receptor. The uptake and degradation of ^{125}I-labeled HTG-VLDL$_1$ is suppressed up to 80–90% by preincubation of the cells with sterols, acetyl LDL, or β-VLDL, indicating that this process is not via the irrepressible chylomicron remnant (apoE) receptor. Chylomicrons, HTG-VLDL$_1$, and thrombin-treated HTG-VLDL$_1$—but not normal VLDL$_1$, β-VLDL, LDL, or acetyl LDL—produce massive triglyceride accumulation (10–20-fold mass increases in 4 hr) in P388D$_1$ macrophages. Triglyceride loading is not enhanced by heparin, indicating that lipoprotein lipase is not involved under these conditions. The P388D$_1$ cell line cultured as described herein represents an ideal model system for studying interactions of lipoproteins with the β-VLDL receptor.—**Gianturco, S. H., S. A. Brown, D. P. Via, and W. A. Bradley.** The β-VLDL receptor pathway of murine P388D$_1$ macrophages. *J. Lipid Res.* 1986. **27:** 412–420.

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Very low density lipoproteins (VLDL) from hypertriglyceridemic (HTG) subjects and chylomicrons are the only native human plasma lipoproteins known to rapidly convert macrophages into foam cells in vitro via a specific receptor pathway (1). The β-VLDL receptor, the responsible receptor (2), is genetically distinct from the LDL receptor (3, 4). It is also present in other cells of reticuloendothelial origin, such as endothelial cells (4) and arterial foam cells (5), but not in non-macrophage cells (for a recent review, see ref. 6). This alternate pathway for disposal of large triglyceride-rich lipoproteins may produce the foam cells that occur in some forms of hypertriglyceridemia (7).

This pathway was first identified in resident murine peritoneal macrophages by its interaction with the diet-induced abnormal cholesteryl ester-rich β-migrating VLDL from cholesterol-fed dogs (β-VLDL) (2). Uptake of β-VLDL triggers a sequence of events analogous to that which follows uptake of low density lipoproteins (LDL) via the LDL receptor. Internalized particles are hydrolyzed in lysosomes, and excess, released cholesterol is re-esterified by acyl-coenzyme A:cholesteryl acyl transferase (ACAT), with oleate as the preferred fatty acid, and stored as cytoplasmic cholesteryl ester droplets. β-VLDL from other cholesterol-fed animals produce saturable cholesterol reesterification in macrophages from murine, rabbit, monkey, and human sources via this receptor pathway (8). By contrast, VLDL, LDL, and HDL from normal animals and humans fail to cause cholesteryl ester accumulation in unstimulated macrophages of any source (6).

We have demonstrated that HTG-VLDL bind with high affinity to the β-VLDL receptor of fresh resident murine peritoneal macrophages (1). After uptake and degradation of HTG-VLDL, the cells acquire morphologic characteristics of foam cells. The major lipid that accumulates in macrophages exposed to HTG-VLDL or chylomicrons is triglyceride, and not cholesteryl ester. Analogous to the incorporation of exogenous oleate into cellular cholesteryl ester after uptake of β-VLDL, oleate incorporation into cellular triglyceride was stimulated by incubation with HTG-VLDL or chylomicrons. Thus the lipid that accumulates in macrophages after receptor-mediated uptake of a lipoprotein reflects the predominant lipid trans-

Abbreviations: HTG-VLDL$_1$, hypertriglyceridemic very low density lipoproteins; VLDL$_1$, S_f 100–400; VLDL$_2$, S_f 60–100; VLDL$_3$, S_f 20–60; LDL, low density lipoproteins; apoE, apolipoprotein E; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecylsulfate.

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ported by that lipoprotein (1, 6). VLDL, LDL, and HDL from normal humans, in contrast to HTG-VLDL and β-VLDL, failed to stimulate oleate incorporation into either cellular cholesteryl ester or triglyceride (1).

The ligands for the β-VLDL receptor remain to be defined. Studies in murine peritoneal macrophages in several laboratories indicate that neither apoE alone nor apoB-100 alone is sufficient for binding (2, 9). Definitive studies for the identification of binding determinants are complex and require large numbers of macrophages, preferably with few or no LDL receptors. Because of the potential importance of this receptor in several clinicopathological phenomena (foam cell accumulation, atherosclerosis, hypertriglyceridemia), we sought a long-term macrophage line for use as a model system to facilitate studies of the β-VLDL receptor pathway. The line P388D_1 was an ideal source for the study and isolation of the acetyl LDL receptor (10, 11). These cells contain very low levels of the LDL receptor without induction (10), they do not express the chylomicron remnant receptor as do human monocyte-macrophages (12) as reported here, and, finally, they do not secrete apoE (13); they seemed, therefore, potentially well suited for studying the β-VLDL receptor pathway. Because both β-VLDL and HTG-VLDL can also bind to the LDL receptor, the presence of high levels of LDL receptors on human monocyte-macrophages and on many long-term macrophage lines introduces ambiguity into the interpretation of direct and competitive uptake and degradation studies designed to identify binding determinants for the β-VLDL receptor.

We now report that the characteristics of the β-VLDL receptor pathway in the murine macrophage line P388D_1 are like those previously described in freshly isolated, unstimulated murine peritoneal macrophages. Moreover, since these cells do not appear to secrete lipoprotein lipase under our culture conditions, cellular triglyceride accumulation can be used as an intracellular end point to monitor receptor-mediated uptake of triglyceride-rich particles.

MATERIALS AND METHODS

Cells and cell culture

The murine macrophage cell line P388D_1 was obtained from the Salk Institute Cell Repository and was cultured in RPMI-1640 (high glucose) supplemented with 1% glutamine, 10% fetal bovine serum (Gibco), 100 μg/ml penicillin, and 100 units/ml streptomycin (10). Cells were maintained in 100-mm dishes in a humidified incubator (5% CO_2) at 37°C.

For experimental purposes, approximately 3.7×10^5 cells/dish were seeded into dishes (60 × 15 mm) in 2 ml of complete medium. Cultures were refed 24 hr later with complete medium, and on the second day the medium was removed. After washing twice with saline, the cells were incubated in 2 ml of serum-free RPMI-1640 containing the desired concentration of lipoprotein, as indicated in the figures and tables.

For binding studies, lipoproteins were iodinated by a modification of the iodine monochloride method of McFarland (14). Free iodine was removed by gel filtration and extensive dialysis. Samples were filtered (0.45 μm Millex) immediately before use; specific activities ranged from 35 to 200 cpm per ng protein. Less than 10% of the label was extractable into organic solvent. Duplicate dishes of cells and empty dishes were incubated with labeled lipoproteins alone and in the presence of indicated quantities of unlabeled lipoprotein at 4°C or 37°C for up to 4 hr as indicated in the figure legends. After 6 hr at 37°C, the HTG-$VLDL_1$ particles denature in the absence of albumin or serum and show greatly increased nonspecific binding to the control dishes that contain no cells. Since inclusion of albumin, fetal calf serum, or lipoprotein-deficient serum to stabilize VLDL diminishes cellular triglyceride accumulation and introduces soluble, transferable apoproteins that affect the binding and uptake of added VLDL, all experiments were conducted at 2 to 4 hr of incubation in the absence of serum components other than lipoproteins. Total cell-associated radioactivity (representing both surface-bound and internalized) was determined after the cells were washed five times with chilled buffer containing 2 mg of albumin/ml and once with albumin-free buffer (15). The amount of non-iodide, non-lipid, trichloroacetic acid-soluble radioactivity in the medium was used as a measure of iodinated lipoprotein degradation (15). There was no evidence of deiodination of ^{125}I-labeled monotyrosine following the cellular degradation of radiolabeled apoproteins. Each value was corrected by subtracting the amount "bound" or degraded in control dishes that contained no cells. Specific binding, uptake, and degradation curves were calculated by subtracting the curve generated by plotting the amount of ^{125}I-labeled lipoprotein processed by cells in the presence of excess unlabeled homologous lipoprotein (these plots were linear) from the curve representing the amount bound in the absence of unlabeled lipoproteins (curvilinear), as in Fig. 1.

Triglyceride mass was determined using an enzymatic kit (Boehringer Mannheim, cat. no. 701912). To determine cellular triglyceride mass, duplicate 60-mm dishes containing cells (and no cells, for blanks) were incubated with the indicated quantities of lipoproteins for 4 hr at 37°C and washed extensively with albumin-containing buffer as in the binding studies. The lipids were then extracted with hexane–isopropanol 3:2 (v/v). The solvent was evaporated under nitrogen, and lipids were resolubilized in 0.5 ml of the kit reagent containing Triton X-100 (10 μl/ml reagent). Cholesterol and cholesteryl ester masses were determined using an enzymatic method (16).

Cells for oil-red O staining were grown, incubated with lipoproteins, and extensively washed as described for

binding studies. The cells were fixed with 60% isopropyl alcohol and stained with oil-red O and hematoxylin as described previously (1). Before examination by light microscopy, cover slips were placed on the stained cells using a 50% solution of polyvinylpyrrolidone as an aqueous mounting medium.

Lipoproteins

Plasma was obtained either from fasting subjects with normal lipid values for isolation of normal VLDL, LDL, and lipoprotein-deficient serum (LPDS) or from fasting subjects with types 3, 4, and 5 lipoprotein profiles for HTG-VLDL and chylomicrons. The diagnoses were based on commonly used criteria (7). Lipoproteins for cell studies were isolated (17) and the VLDL were subfractionated (18) from fresh plasma containing 1 mM EDTA, 1 mM NaN_3, 10 μM phenylmethylsulfonyl fluoride (Sigma), and 50 units of Trasylol/ml, as previously detailed (19, 20). Chylomicrons were isolated by a 20-min centrifugation in an SW 41 rotor at 12,000 rpm and 23°C and washed three times through saline containing 1 mM EDTA, pH 7.4. LDL were isolated at d 1.03–1.05 g/ml and washed at each density. β-VLDL were isolated from cholesterol-fed rabbits as previously described (1). Total protein contents of the lipoproteins were obtained either by a modification (21) of the method of Lowry et al. (22) or by amino acid analysis. Characteristics of VLDL subclasses so isolated are detailed elsewhere (1, 19, 23).

HTG-VLDL were incubated with 200 units of purified human α-thrombin/mg of apoprotein for 2 hr at 37°C in 0.15 M NaCl, 20 mM Tris, pH 7.4, 10 mM $CaCl_2$ (buffer), or with buffer alone (control). After incubation at 37°C for 2 hr, aliquots of the thrombin-treated and control lipoproteins were then recentrifuged through a discontinuous salt gradient (d 1.05–1.006 g/ml) to reisolate the HTG-$VLDL_1$. Thrombin-treated, but not control, HTG-$VLDL_1$ no longer suppress HMG-CoA reductase activity or effectively compete with LDL binding in normal human fibroblasts due to cleavage of the thrombin-accessible apoE, as determined by immunochemical blotting (24, 25).

RESULTS

Saturable, high-affinity uptake and degradation of iodinated HTG-$VLDL_1$

HTG-$VLDL_1$ isolated from the plasma of subjects with hypertriglyceridemia types 3, 4, and 5 interact with $P388D_1$ macrophages in a high-affinity, curvilinear manner (upper curve, **Fig. 1**) similar to that observed in fresh murine peritoneal macrophages (1). When incubated in the presence of excess unlabeled HTG-$VLDL_1$, the high-affinity binding, uptake, or degradation of iodinated HTG-$VLDL_1$ is abolished; a plot of the residual, nonspecific binding of iodinated HTG-$VLDL_1$ that occurs in the presence of excess unlabeled lipoprotein is linear. Cell-associated iodinated HTG-$VLDL_1$ is illustrated in Fig. 1; similar curves were obtained for degradation. Half-maximal and maximal specific uptake (Fig. 1) and degradation (data not shown) occur at HTG-$VLDL_1$ levels of ~1 and 8 μg HTG-$VLDL_1$ protein/ml, respectively. The amount of cell-associated ^{125}I-labeled HTG-$VLDL_1$, both total and specific, is at least fourfold greater at 37°C than at 4°C in a standard 2-hr incubation, indicating uptake and intracellular accumulation at 37°C. Similar values were obtained with HTG-$VLDL_1$ from four different subjects in separate experiments.

To demonstrate that the receptor-mediated degradation of HTG-$VLDL_1$ occurs in lysosomes, as in peritoneal macrophages (1), we determined the effects of chloroquine, an inhibitor of lysosomal degradation, on both total and nonspecific uptake and degradation of HTG-$VLDL_1$. Chloroquine inhibited both total and specific degradation (**Fig. 2**) in a dose-dependent manner, but had little effect on cellular uptake (not shown). Chloroquine at 20 μM inhibited specific degradation by approximately one-half, and 50 μM chloroquine completely inhibited specific degradation. At the higher chloroquine concentration, specific cellular uptake was slightly enhanced, as is expected when degradation is inhibited but uptake is not.

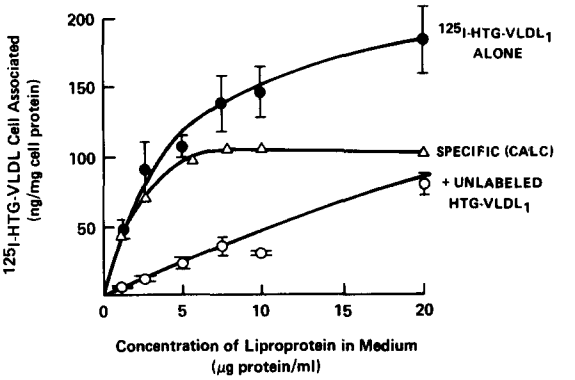

Fig. 1. Uptake of ^{125}I-labeled HTG-$VLDL_1$ by the β-VLDL receptor of $P388D_1$ macrophages. Cells were grown to approximately 60% confluency in 60-mm dishes in complete medium for 2 days and washed. Medium containing the indicated quantities of ^{125}I-labeled HTG-$VLDL_1$ (69 cpm/ng), in the absence (●) or in the presence (○) of unlabeled homologous HTG-VLDL, 50 μg protein/ml, was added to triplicate dishes and incubated in a humidified CO_2 incubator for 2 hr at 37°C. Cells were washed extensively and dissolved in 2.0 ml of 0.1 N NaOH. Radioactivity associated with the cell lysate represents ^{125}I-labeled HTG-$VLDL_1$ uptake. The VLDL were obtained from a subject with type 5 hypertriglyceridemia. Each data point represents the mean ± SD of values from three dishes, expressed as ng of ^{125}I-labeled HTG-VLDL per mg of cell protein. The calculated difference between the curves representing uptake of ^{125}I-labeled HTG-$VLDL_1$ in the absence (●) and in the presence (○) of unlabeled HTG-$VLDL_1$ represents the specific uptake (△). The average protein content per dish was 483 μg.

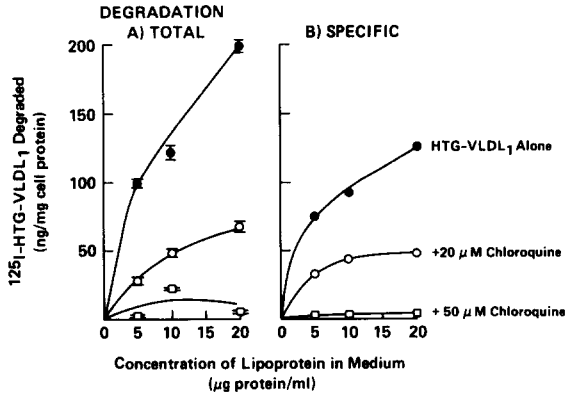

Fig. 2. Effect of chloroquine on the degradation of ^{125}I-labeled HTG-VLDL$_1$ in P388D$_1$ macrophages. Cells were grown in complete medium for 2 days and washed. Medium containing the indicated quantities of ^{125}I-labeled HTG-VLDL$_1$ (62 cpm/ng) and chloroquine in the absence or presence of unlabeled HTG-VLDL$_1$ (100 μg/ml) was added to duplicate dishes and incubated at 37°C. After 4 hr the degradation of ^{125}I-labeled HTG-VLDL$_1$ was determined. Each total degradation data point represents the average of values from duplicate dishes; range is indicated by bars. Specific degradation was calculated as described in Methods and the legend to Fig. 1. ^{125}I-labeled HTG-VLDL alone (●), +20 μM chloroquine (○), and +50 μM chloroquine (□).

Specificity of binding

Competition studies indicate that the specificity of the binding, uptake, and degradation of HTG-VLDL$_1$ in P388D$_1$ macrophages is the same as that observed in the fresh murine peritoneal macrophages (1, 26). β-VLDL, HTG-VLDL$_1$, and chylomicrons compete efficiently for the uptake and degradation of HTG-VLDL$_1$, but neither LDL nor acetyl LDL effectively compete (**Fig. 3**). Similar results were obtained in six competition experiments using HTG-VLDL$_1$ from six hypertriglyceridemic subjects. These competitive uptake and degradation studies indicated that under these conditions the triglyceride-rich lipoproteins and β-VLDL bind to common receptors that are distinct from either the classic LDL receptor or the acetyl LDL receptor.

To demonstrate further that the receptor on P388D$_1$ macrophages to which HTG-VLDL$_1$ binds is not the classic LDL receptor, we abolished the binding of HTG-VLDL$_1$ to the LDL receptor by treating it with purified human α-thrombin (24, 25). The binding of large HTG-VLDL (S_f > 60) to the LDL receptor is mediated by an accessible conformation of apoE that can be specifically cleaved by thrombin into two major fragments; thrombin cleavage of this apoE abolishes the binding of HTG-VLDL$_1$ to the LDL receptor (24, 25). By contrast, thrombin-treated HTG-VLDL$_1$ bind to the β-VLDL receptor of fresh murine peritoneal macrophages as well as or better than the native HTG-VLDL$_1$ (26). As seen in Fig. 3, thrombin-treated HTG-VLDL$_1$ also compete effectively for the β-VLDL receptor of P388D$_1$ macrophages. The same thrombin-treated HTG-VLDL$_1$ were tested in fibroblasts as a control to show that LDL receptor binding determinants were indeed lost upon thrombin treatment, as previously published (24, 25). Likewise, the cleavage of the accessible apoE was monitored by immunochemical electrophoretic blotting as previously published (24, 25). This experiment has been repeated more than 10 times with no loss of binding activity of HTG-VLDL$_1$ in P388D$_1$ cells upon thrombin treatment. The failure of thrombin treatment to diminish the competitive uptake and degradation of HTG-VLDL$_1$ (Fig. 3) while abolishing LDL receptor-mediated uptake by fibroblasts (24, 25) indicates that in the P388D$_1$ macrophages the ^{125}I-labeled HTG-VLDL are binding not to the classic LDL receptor but to a distinct receptor that recognizes chylomicrons, HTG-VLDL, β-VLDL, and thrombin-treated HTG-VLDL$_1$, but not LDL.

A recent report demonstrates that human monocyte-macrophages contain a receptor for chylomicron remnants (12) analogous to the hepatic apoE receptor (27). This receptor, in contrast to the β-VLDL receptor, is not down-regulated by sterols, acetyl LDL, or β-VLDL. Therefore we asked: can the uptake of HTG-VLDL by P388D$_1$ cells be down-regulated? Preincubation of cells with cholesterol plus 25-hydroxycholesterol, β-VLDL, or acetyl LDL inhibited the specific degradation of iodinated

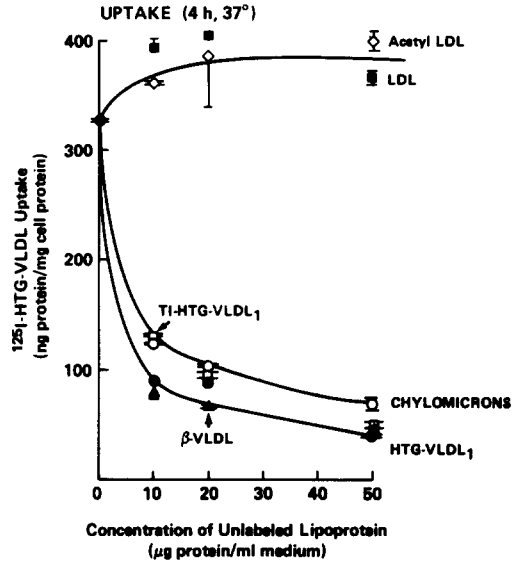

Fig. 3. Specificity of the β-VLDL receptor pathway of P388D$_1$ macrophages. Cells were grown as described in the legend of Fig. 1. Two ml of medium containing ^{125}I-labeled HTG-VLDL$_1$, 2 μg protein/ml, 104 cpm/ng, alone and with the indicated quantities of lipoproteins, was added to duplicate dishes and incubated for 4 hr at 37°C. The cells were washed extensively with albumin-containing buffer and dissolved in 0.1 N NaOH; radioactivity of the cell lysate represents uptake of ^{125}I-labeled HTG-VLDL$_1$, and is plotted as a function of the amount of unlabeled lipoprotein protein present in the medium. Each point represents the average of values from duplicate dishes; range is indicated by bars. The HTG-VLDL (●) were obtained from a subject with type 4 hypertriglyceridemia. Normal LDL (■), acetyl LDL (◇), chylomicrons (○), β-VLDL (▲), and thrombin-treated HTG-VLDL$_1$ (□) were prepared as described in Methods.

HTG-VLDL by 80 to 90% (**Fig. 4**), indicating that the degradation observed without preincubation is indeed due to the regulatable β-VLDL receptor rather than to the unregulatable chylomicron remnant receptor.

Lipid accumulation in P388D$_1$ cells

Chylomicrons and HTG-VLDL$_1$ from type 4 and 5 subjects—but not normal VLDL$_1$, β-VLDL, LDL, or acetyl LDL—produce massive triglyceride accumulation in P388D$_1$ macrophages (**Fig. 5**, **Fig. 6**, and **Table 1**); β-VLDL produce cholesteryl ester accumulation. As reported previously (10), acetyl LDL, but not LDL, cause massive cholesteryl ester accumulation via the acetyl LDL receptor pathway in P388D$_1$ cells (Table 1).

The amount of cellular triglyceride accumulated by P388D$_1$ macrophages when exposed to HTG-VLDL from type 4 and 5 subjects in only 4 hr is dramatic. HTG-VLDL$_1$ at 25 μg of HTG-VLDL$_1$ protein/ml produce a 10–20-fold increase in cellular triglyceride, from 10 to 20 μg of triglyceride/mg cell protein up to 200 μg of triglyceride/mg cell protein. Triglyceride accumulates in a curvilinear manner, with half-maximal and maximal values between 5–10 and 25–40 μg of protein/ml (Fig. 5). Normal VLDL $S_f > 60$ fail to induce this pronounced cellular triglyceride engorgement, when compared on the basis of either protein (Fig. 5) or triglyceride (Fig. 6) content. In experiments not shown, HTG-VLDL$_1$ produced the same or greater triglyceride accumulation after thrombin treatment to abolish binding to the LDL receptor. Triglyceride accumulation experiments with similar results have been performed 18 times with VLDL S_f > 60 from seven hypertriglyceridemic subjects, VLDL $S_f > 60$ from fifteen normal subjects, and chylomicrons from four hypertriglyceridemic subjects.

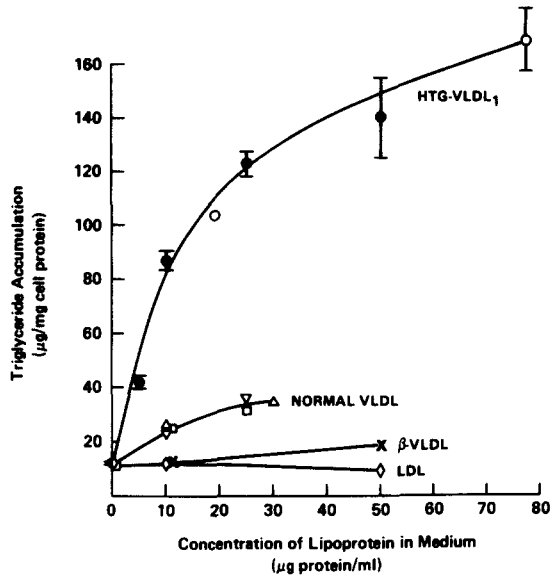

Fig. 5. Effects of lipoproteins on triglyceride accumulation in P388D$_1$ macrophages. Cells were grown as described in Methods. Indicated amounts of lipoproteins were added to duplicate dishes and incubated for 4 hr at 37°C. The cells were washed extensively with albumin-containing buffer and the lipids were extracted in situ with hexane–isopropanol, as described in Methods. Normal VLDL S_f 60–400 and LDL preparations were from normolipidemic donors and HTG-VLDL$_1$ were from a patient with type 5 hypertriglyceridemia. Each point represents the value from duplicate dishes, except for the incubations with normal VLDL, which are single determinations of VLDL from three different subjects due to the paucity of VLDL $S_f > 60$ in normal subjects. The triglyceride accumulated by the cells is plotted as a function of the amount of lipoprotein protein present in the medium. The average cellular protein content per dish was 493 μg. HTG-VLDL$_1$ (●); normal VLDL (□, △, ▽); LDL (◇); and rabbit β-VLDL (X).

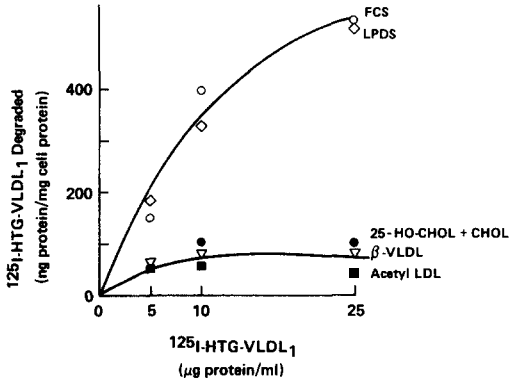

Fig. 4. Effects of preincubation on expression of the β-VLDL receptor. Cells were grown as described except that 24 hr prior to incubation of the cells with the ^{125}I-labeled HTG-VLDL$_1$, the cells were exposed to RPMI-1640 medium containing fetal calf serum (FCS; regular complete medium) (○); 10% lipoprotein-deficient human serum (LPDS) (◇); cholesterol (chol), 16 μg/ml, plus 25-hydroxycholesterol (25-HO-chol), 1 μg/ml (●); cholesteryl ester-rich β-VLDL, 25 μg protein/ml (▽); or acetyl LDL, 21 μg protein/ml (■). The medium was then removed, the cells were washed extensively and then incubated for 4 hr with the indicated quantities of ^{125}I-labeled HTG-VLDL. TCA-soluble, non-iodide radioactivity, from duplicate dishes represents degradation; each value is the average.

To determine whether the massive, rapid triglyceride accumulation in P388D$_1$ cells exposed to HTG-VLDL or chylomicrons is mediated in part by lipoprotein lipase (28), we incubated the cells with HTG-VLDL$_1$ in the presence and absence of heparin (**Table 2**). Heparin enhances the lipoprotein lipase-mediated triglyceride accumulation by preadipocytes incubated with VLDL (29), stimulates the release of lipoprotein lipase from ascites macrophage lines (28), and enhances triglyceride accumulation in murine peritoneal or J774 macrophages in long-term (24–48 hr) incubations (30). In two separate experiments, heparin at levels known to enhance lipase activity failed to enhance the HTG-VLDL$_1$-induced triglyceride accumulation in P388D$_1$ macrophages in 4 hr (Table 2). Moreover, normal VLDL do not induce massive triglyceride accumulation in P388D$_1$ macrophages even though both normal VLDL and HTG-VLDL are good substrates for lipoprotein lipase. These observations indicate that, in these short-term experiments, lipoprotein

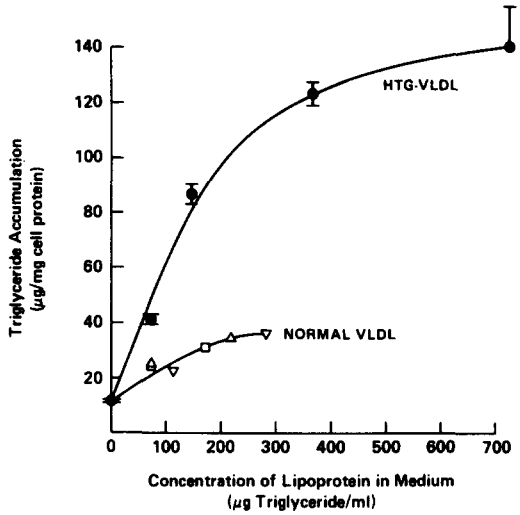

Fig. 6. Effects of HTG-VLDL and normal VLDL on triglyceride accumulation in P388D$_1$ macrophages. The cells, lipoproteins, and experimental conditions are those described in the legend of Fig. 5. Cellular triglyceride accumulation is plotted as a function of the amount of lipoprotein triglyceride present in the medium.

lipase contributes little, if any, to the observed triglyceride accumulation induced by HTG-VLDL and chylomicrons.

The lipid engorgement of P388D$_1$ cells induced by a 4-hr incubation with chylomicrons, HTG-VLDL, β-VLDL, and acetyl LDL was readily visualized after staining of the cells with the neutral lipid dye oil-red O (data not shown). Incubation with normal VLDL at triglyceride concentrations equal to those of HTG-VLDL or chylomicrons resulted in few oil-red O droplets, consistent with the minimal triglyceride mass increase measured chemically. Likewise, LDL failed to induce visible lipid accumulation. The lipid inclusions were apparent under phase contrast in living cells.

DISCUSSION

Uptake of triglyceride-rich lipoproteins by the β-VLDL receptor pathway may be responsible for the accumulation of lipid-filled foam cells and atherosclerosis associated with some forms of hypertriglyceridemia (7). For example, the foam cells from eruptive xanthomas in hypertriglyceridemic diabetics are filled with triglycerides (31). When lesions regress upon insulin treatment with consequent lowering of plasma triglyceride levels, the triglycerides rapidly diminish, leaving cholesterol as the predominant lipid (31). Perhaps a similar process occurs in the genesis of arterial foam cells in hypertriglyceridemia.

The maximal binding, uptake, degradation, and triglyceride accumulation in macrophages via the β-VLDL receptor occur at or below the HTG-VLDL$_1$ levels that occur in the plasma of many hypertriglyceridemic subjects. Thus there is sufficient HTG-VLDL$_1$ present in these subjects' plasma to saturate β-VLDL receptors present on monocytes, macrophages, or endothelial cells exposed to plasma. Cellular catabolism of HTG-VLDL$_1$ by the β-VLDL receptor (1), the LDL receptor (32), and/or the hepatic apoE receptor (33) may account for the disappearance of large HTG-VLDL directly from the plasma that has been observed in hypertriglyceridemic subjects (34).

In this report we present several lines of evidence indicating that murine P388D$_1$ macrophages are a good

TABLE 1. Effects of lipoprotein on the lipid composition of P388D$_1$ macrophages

Lipoprotein	Concentration in Medium				Cellular Lipid Content		
	Protein	TG	Chol	CE	TG	Chol	CE
	μg/ml				*μg lipid/mg cell protein*		
None	0	0	0	0	15.4	16.3	0.6
LDL	10	2	11.4	22	11.2	10.7	1.0
	40	8	45.6	86	8.0	9.9	3.9
HTG-VLDL$_1$	10	120	10.0	4	45.6	19.6	0
	40	481	40.0	18	96.1	19.1	6.5
Chylomicrons[a]	2.8	127	9.7	6	45.7	26.1	1.4
	11	510	39.0	21	102.0	30.0	16.0
β-VLDL	40	13	191.6	351	14.1	32.0	16.4
Acetyl LDL	10	2	11.6	26	6.5	20.6	20.1
	40	10	46.4	105	5.5	27.0	36.3

Cells were grown as described in the legend to Fig. 1. Each dish received 2 ml of complete medium containing the indicated lipoproteins and was incubated at 37°C for 4 hr. The lipoprotein concentrations are given in terms of micrograms lipoprotein protein, triglyceride (TG), cholesterol (Chol), and cholesteryl ester (CE) per ml. The LDL preparation was from a normal donor. The HTG-VLDL$_1$ and chylomicron preparations were from a patient with type 5 hypertriglyceridemia. Acetyl LDL were prepared from normal LDL. β-VLDL were isolated from cholesterol-fed rabbits and tested in a separate experiment. Intracellular lipid concentrations are expressed as micrograms lipid per milligram cell protein, and each value is the average of values from duplicate dishes, which varied by <9%.

[a]Chylomicron protein content estimation from average composition reported (38) using a TG/Prot = 45. TG was assayed enzymatically as described in Methods.

TABLE 2. Effect of heparin on triglyceride accumulation in P388D$_1$ macrophages

Lipoprotein	Cellular Triglyceride	
	Heparin	
	0	10 U/ml
	μg/mg	
None[a]	12.4[b]	8.5
HTG-VLDL$_1$ 20 μg/ml	34.7	27.1
HTG-VLDL$_1$ 40 μg/ml	95.5	90.3

[a]The cells were grown and incubated for 4 hr in the absence or presence of two levels of HTG-VLDL$_1$ in the absence or presence of heparin before cellular triglycerides were determined as described in Methods and the legends to Figs. 5 and 6.

[b]Cellular triglyceride concentrations are expressed as μg triglyceride/mg cell protein. Each data point is the average of values from duplicate dishes, which varied <6%.

model system for studying the β-VLDL receptor pathway. First, as demonstrated previously (10), this long-term cell line has very few LDL receptors without induction, simplifying interpretation of the binding studies. Second, the specificity of the receptor pathway in the long-term macrophage line is identical to that in fresh murine peritoneal macrophages. Chylomicrons, HTG-VLDL$_1$, thrombin-treated HTG-VLDL (which do not bind to the LDL receptor), and β-VLDL from cholesterol-fed rabbits bind with high affinity to this receptor and rapidly produce massive lipid accumulation. Competition studies indicate that these lipoproteins mutually compete, but neither acetyl LDL nor normal LDL effectively compete for the uptake of either HTG-VLDL$_1$ or β-VLDL by these cells. These studies indicate that HTG-VLDL$_1$ are not being taken up by either the acetyl LDL or the LDL receptor pathway. Furthermore, this pathway, in sharp contrast to the chylomicron remnant (apoE) receptor, is efficiently down-regulated by preincubating cells with sterols, cholesteryl ester-rich β-VLDL, or acetyl LDL, conditions that load the cells with cholesteryl esters. This indicates that uptake of HTG-VLDL under these conditions is not via the chylomicron remnant (apoE) receptor. Third, as seen in murine peritoneal macrophages, the receptor-mediated degradation of HTG-VLDL$_1$ by P388D$_1$ macrophages is inhibited by chloroquine, indicating that degradation occurs in lysosomes. Fourth, the pattern of lipid accumulation induced in P388D$_1$ cells by lipoproteins is like that observed in fresh murine peritoneal macrophages. HTG-VLDL and chylomicrons produce massive, saturable triglyceride accumulation. Both cholesteryl ester-rich β-VLDL and acetyl LDL produce massive cholesteryl ester accumulation. LDL produce no lipid accumulation even when incubated with the cells for prolonged periods. Likewise, VLDL from fasting normal subjects failed to induce either triglyceride or cholesteryl ester accumulation in these cells.

For several reasons, the massive, rapid triglyceride accumulation induced in P388D$_1$ cells by HTG-VLDL$_1$ or chylomicrons does not appear to be mediated by lipoprotein lipase. First, normal VLDL, which are competent substrates for lipoprotein lipase, fail to induce cellular triglyceride engorgement when compared on the basis of either protein or triglyceride content (Figs. 5 and 6). Second, heparin, which is known to enhance lipoprotein lipase-mediated cellular triglyceride accumulation, failed to enhance the accumulation of triglyceride when the cells were incubated with HTG-VLDL$_1$ (Table 2). Moreover, no lipoprotein lipase activity could be detected in P388D$_1$-conditioned media in a sensitive assay (M. Rohde, personal communication). We conclude that, under the conditions of these short-term incubations, lipoprotein lipase contributes little if any to the observed triglyceride accumulation induced by HTG-VLDL$_1$ and chylomicrons. Thus cellular triglyceride accumulation can serve as an intracellular end point for the β-VLDL receptor pathway in P388D$_1$ cells under these conditions.

In addition, P388D$_1$ macrophages are well suited for studying the binding determinants for the β-VLDL receptor because these cells, unlike human monocyte-macrophages and cholesteryl ester-loaded peritoneal macrophages, do not secrete apoE (13 and D. P. Via, unpublished observations). Secretion of apoE could cloud the interpretation of binding studies since apoE appears to mask domains involved in the binding of HTG-VLDL to the β-VLDL receptor (26) and mediates binding to the LDL receptor (24, 25) and the chylomicron remnant receptor (12, 27).

The specific requirements for binding and uptake of a lipoprotein via the β-VLDL receptor have not been pinpointed. The difficulties in identifying the receptor recognition factors arise from the presence of several different lipoprotein receptors and mechanisms of uptake of lipoproteins by macrophages. There are, at least, three distinct receptors on macrophages that recognize β-VLDL: the LDL receptor (35), the β-VLDL receptor (2), and the chylomicron remnant/apoE receptor (12). Depending upon cell type, cell isolation, and culture conditions used, differing levels of each of these receptors can be expressed. When either or both the LDL receptor and the chylomicron remnant receptor are present, uptake of β-VLDL could occur via these receptors and be mediated by apoE. HTG-VLDL are known to bind to the LDL receptor via apoE (24, 25), and probably to the chylomicron remnant (apoE) receptor as well. By contrast, chylomicrons do not appear to interact with either the LDL receptor (36) or the chylomicron remnant receptor (12, 33), but do interact with the β-VLDL receptor (3 and this report). Likewise, thrombin-treated HTG-VLDL $S_f > 60$ do not bind to the LDL receptor (24, 25) but bind to the β-VLDL receptor of macrophages with affinity equal or enhanced to that of the native particle (26 and this report). We conclude that the thrombin-accessible apoE is not necessary

for uptake by this receptor but can modulate binding by masking domains involved in binding, since reincorporation of apoE into thrombin-treated HTG-VLDL can reverse or even inhibit the thrombin-enhanced uptake by macrophages isolated and cultured under conditions where there are few-to-no LDL or chylomicron remnant/apoE receptors (26). We suggest that domains in apoB species other than apoB-100 are recognized by the receptor, either in apoB-48 (3, 37) or apoB species present in native HTG-VLDL or created by protease cleavage (25, 26). An apoB domain may be sufficient alone or receptor recognition may require participation of another component, such as a second apoB domain or a region of the thrombin-inaccessible apoE.

The P388D$_1$ murine macrophages cultured as described in this report represent an ideal model system for studying the interactions of various lipoproteins with the β-VLDL receptor, and we anticipate that their use will facilitate identification of the factors necessary for binding to and uptake via the β-VLDL receptor.

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