Heparan Sulfate Proteoglycan-Mediated Uptake of Apolipoprotein E−Triglyceride-Rich Lipoprotein Particles: A Major Pathway at Physiological Particle Concentrations†

Maysoon Al-Haideri,‡ Ira J. Goldberg,§ Narmer F. Galeano,‡ Anne Gleeson,‡ Tikva Vogel,|| Marian Gorecki,|| Stephen L. Sturley,‡,⊥,▽ and Richard J. Deckelbaum*,‡,⊥

Departments of Pediatrics, Medicine, and Physiology and The Institute of Human Nutrition, Columbia University, New York, New York 10032, and Biotechnology General Ltd., Rehovot, Israel

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ABSTRACT: We explored potential mechanisms of non-low-density lipoprotein (LDL) receptor-mediated uptake of triglyceride-rich particles (TGRP) in the presence of apolipoprotein E (apo E). Human fibroblasts were incubated with model intermediate-density lipoprotein- (IDL-) sized TGRP (10−1000 μg of neutral lipid/mL) containing apo E. The extent of receptor-mediated uptake of TGRP was assessed with (a) an anti-apo E monoclonal antibody, which blocks receptor interaction; (b) incubation with heparin; (c) normal vs LDL receptor-negative fibroblasts; and (d) receptor-associated protein (RAP) to determine the potential contribution of LDL receptor-related protein (LRP). Cell surface heparan sulfate proteoglycan- (HSPG-) mediated uptake was examined with or without the addition of heparinase and heparitinase to cell incubation mixtures. At low particle concentrations ($\leq$100 μg of neutral lipid/mL), almost all apo E−TGRP uptake was via the LDL receptor. At higher particle concentrations, within the physiologic range ($>$250 μg of neutral lipid/mL), most ($\geq$60%) particle uptake and internalization was via HSPG-mediated pathways. This HSPG pathway did not involve classical lipoprotein receptors, such as LRP or the LDL receptor. These data suggest that in peripheral tissues, such as the arterial wall, apo E may act in TGRP as a ligand for uptake not only via the LDL receptor and LRP pathways but also via HSPG pathways that are receptor-independent. Thus, at physiologic particle concentrations apo E−TGRP can be bound and internalized in certain cells by relatively low affinity but high capacity HSPG-mediated pathways.

Apolipoprotein E (apo E) binds to a number of lipoprotein receptors including the LDL[1] receptor, and the LDL receptor-related protein (LRP) (Mahley, 1988; Ji et al., 1993). In addition to its role in receptor-mediated endocytosis, apo E may mediate uptake of TGRP by non-LDL receptor pathways, especially in nonhepatocyte cells such as macrophages (Hussain et al., 1989). In this regard, recent evidence shows that apo E in TGRP binds to cell surface HSPG, presumably via the heparin binding sites on apo E (Ji et al., 1993). This binding to HSPG may be particularly important in the liver, where apo E (a) is secreted by hepatocytes, (b) binds to the cell surface (Hamilton et al., 1990), (c) associates with triglyceride-rich lipoproteins, enabling them to interact more effectively with HSPG, and then (d) acts as a ligand for uptake via the LDL receptor, LRP, or other pathways (Ji et al., 1993; Willnow et al., 1994). These properties of apo E appear to be involved in what has been termed the "secretion−recapture" role of apo E (Hamilton et al., 1990; Mahley & Hussain, 1991; Brown et al., 1991). Another molecule, lipoprotein lipase, binds to HSPG and has also been shown to be a ligand that can enhance cellular binding and uptake of TGRP with or without apo E (Cheng et al., 1981; Cupp et al., 1987; Rumsey et al., 1992).

Apo E is tetrameric in aqueous solution but monomeric on lipid particle surfaces (Funahashi et al., 1989). Apo E has well-defined functional domains; the carboxyl-terminal domain binds to lipid and the amino-terminal domain contains the ligand for binding to the LDL receptor (Wilson et al., 1991). Two heparin binding motifs have been identified on apo E; the first is located near the center of the protein and coincides with the receptor binding domain, and the second is located toward the carboxyl-terminal end of the protein and binds heparin with lower affinity (Mahley et al., 1979). Thus apo E has regions that would make it a suitable ligand for binding to cell surface receptors and to HSPG.

Our previous studies showed that cells internalize substantial amounts of model TGRP in the absence of apo E or other apoproteins, via adsorptive endocytosis pathways that clearly did not involve conventional cell receptors (Granot et al., 1994; Schwiegelshohn et al., 1995). Still, at physiological concentrations of model TGRP (equivalent to plasma

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* Address all correspondence to this author at the Department of Pediatrics, Columbia University College of Physicians and Surgeons, 630 West 168th St., New York, NY 10032. Phone (212) 305-7082; Fax (212) 305-8995.

‡ Department of Pediatrics, Columbia University.

§ Department of Medicine, Columbia University.

|| Biotechnology General Ltd.

⊥ The Institute of Human Nutrition, Columbia University.

▽ Department of Physiology, Columbia University.

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[1] Abbreviations: TGRP, triglyceride-rich particles; apo, apoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate-density lipoprotein; LRP, low-density lipoprotein receptor related protein; HSPG, heparan sulfate proteoglycan; NL, neutral lipid; TG, triglyceride; CE, cholesteryl ester; H/H, heparinase/heparitinase; RAP, receptor-associated protein; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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concentrations of 20−100 mg/dL triglyceride), particle uptake is markedly enhanced by addition of apo E (Granot et al., 1994).

Most studies assessing the binding of lipoprotein particles to high-affinity receptors have been performed at particle concentrations much lower than those occurring physiologically. For this reason, we compared apo E−TGRP uptake at low particle concentrations saturating the LDL receptor with cell uptake at higher, more physiologic concentrations. We hypothesized that as particle concentration increased, cell LDL receptors would be saturated and then non-LDL receptor pathways would contribute more to cellular binding and uptake. Our results support this hypothesis and indicate that in cultured fibroblasts (and macrophages) incubated with low concentrations of particles, TGRP uptake is mediated mainly by the LDL receptor and is not appreciably affected by removal of cell surface HSPG. In contrast, at particle concentrations similar to what is found in plasma and in the extracellular milieu, cell surface HSPG can be a predominant mechanism for binding and internalizing apo E−TGRP.

EXPERIMENTAL PROCEDURES

Materials. Triolein and cholesteryl oleate were purchased from Nu-Chek Prep. Inc. (Elysian, MN). Egg yolk phosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL). Phosphotungstic acid was obtained from Ladd Research Industries (Burlington, VT), and bacitracin was from Sigma Chemical Co. (St. Louis, MO). Heparin was obtained from Elkins-Sinns, Inc. (Cherry Hill, NJ) and had an activity of 1 unit/5.5 μg of heparin. All isotopically labeled compounds were purchased from New England Nuclear (Boston, MA). Heparinase and heparitinase were purchased from Seikagaku American Inc. (Rockville, MD). Bovine serum albumin fraction V was from Sigma Chemical Co. (St. Louis, MO). Cell cultivation materials including glutamine, penicillin, streptomycin, and fetal or standard calf serum were purchased from Gibco/Life Technology Inc. (Grand Island, NY). The enzymatic colorimetric assays to determine triglyceride (triglyceride GPO-PAP test) and cholesteryl ester (cholesterol/HP) were purchased from Boehringer Mannheim (Indianapolis, IN). Sepharose CL-2B was provided by Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Silica gel 60 for TLC was purchased from Merck (Darmstadt, Germany). Purified recombinant apo E_3 produced in *Escherichia coli* was provided by Biotechnology General (Rehovot, Israel). This apo E has been previously shown to exhibit the same in vitro cell binding and in vivo plasma behavior as does native plasma apo E_3 (Vogel et al., 1985). Anti-apo E 1D7 monoclonal antibody was kindly provided by Drs. Ross Milne and Yves Marcel (University of Ottawa) and RAP (the 39 kDa receptor-associated protein) by Dr. Dudley Strickland and colleagues (American Red Cross, Rockville, MD).

Cells. Monolayer cultures of human fibroblasts from normal neonate foreskin or LDL receptor-negative GM 2000 fibroblasts from Coriell Co. (Camden, NJ) were prepared from frozen cells at densities of approximately 2.5×10^4 or 5×10^4 cells/well in plates of 12 or 6 wells respectively. Cells were grown in a humidified incubator (5% CO_2) at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal or standard calf serum, glutamine (292 μg/mL), penicillin (100 units/mL), and streptomycin (100 units/mL) as described elsewhere (Tabas et al., 1985). After the cells attained 80% confluence, LDL receptors were upregulated by cultivation in fresh medium containing 10% (v/v) lipoprotein-deficient serum for 2 days.

Preparation of Lipid Emulsions. Model IDL-sized TGRP were prepared as previously detailed (Schwiegelshohn et al., 1995). Triolein (30−40 mg) and an equal weight of cholesteryl oleate were mixed with twice that amount of egg yolk phosphatidylcholine (1% solution in chloroform). Depending on the purpose of the experiment, either [^{3}H]-cholesteryl hexadecyl ether [cholesteryl-1,2-^{3}H(N)]-, or [^{3}H]-cholesteryl oleate [cholesteryl-1,2,6,7-^{3}H(N)]-, was added in the ratio of 2 μCi/mg of neutral lipid. Because [^{3}H]-cholesteryl ether is not metabolized by mammalian cells, it can be used as a nondegradable marker for particle internalization by cells (Schwiegelshohn et al., 1995).

Analytical Procedures. Triglycerides and cholesteryl oleate were determined with the aid of commercial kits, catalog nos. 450032 and 290319, respectively (Boehringer Mannheim Corp., Indianapolis, IN). Phospholipid was assayed according to Bartlett (1959). Protein was determined by the Lowry method (Lowry et al., 1952).

Characterization of TGRP. Emulsion particles were sized by using a Sepharose CL-2B column (1.6 × 50 cm). Lipoprotein buffer solution (150 mM NaCl and 0.24 mM EDTA, pH 8.4, density 1.006 g/mL) was the eluent, and 1 mL fractions were collected. The particles were detected by measuring absorbance at 280 nm or by radioactivity.

Emulsion particle homogeneity was also examined with the aid of negative staining under a Jeol 1200 EX electron microscope, (Peabody, MA), with the modifications previously described (Fote & Nordhouses, 1986). A 1:100 dilution of emulsion in buffer containing 0.25 mg/mL bacitracin to achieve even coating (Via et al., 1982) was employed. One drop of the diluted emulsion was placed for 30 s on a 200 mesh FormVar carbon-coated grid that was precoated (Mims et al., 1986) with 0.1% BSA. Excess sample was removed with filter paper touched to the edge of the grid. One drop of 2% sodium phosphotungstate (pH 7.2) was then placed on the grid. After removal of excess phosphotungstate, the preparation was permitted to dry in air. Particle size was measured with the aid of a Peak Scale Lupe, Electron Microscopy Sciences (Fort Washington, PA), and ranged between 25 and 35 nm diameter. The emulsion preparations contained no contaminating liposomes.

By column chromatography (Schwiegelshohn et al., 1995), the particles that were used in this study eluted in a region before LDL, similar to the elution position of IDL or remnant-sized particles. The mass ratio of the triolein plus cholesteryl oleate to phospholipid correlated closely with particle size. The ratios measured were between 2:1 and 3:1 (mean 2.3 ± 0.1, $n = 10$), consistent with that for IDL or remnant-sized particles.

Iodination of Apo E. Iodination of apo E was performed according to Bolton and Hunter (1973). Apo E (2 mg) in phosphate buffer at pH 8.0 was added to 1 mCi of N_2-dried *N*-succinimido-3-(4-hydroxy-3-[^{125}I] iodophenyl)propionate and kept at 4 °C for 30 min with manual mixing. The mixture was then dialyzed against a lipoprotein buffer (150 mM NaCl and 0.24 mM EDTA, pH 8.4, density 1.006 g/mL) (Innerarity et al., 1979).

Cell Uptake of Apo E−TGRP. For experiments with apo E, radiolabeled emulsion particles were preincubated with

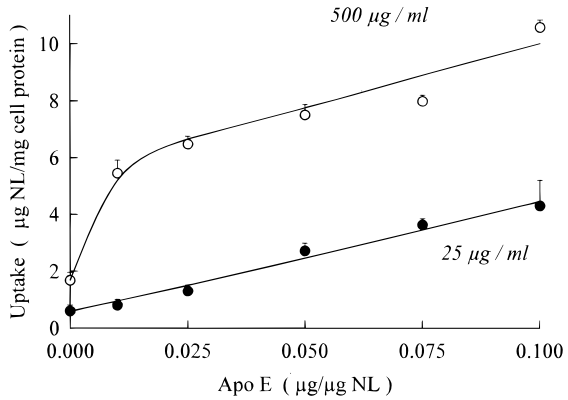

FIGURE 1: Effect of apo E concentrations on TGRP uptake. Normal receptor-positive fibroblasts were incubated at 37 °C for 4 h in media containing increasing amounts of apo E, at low (25 μg of neutral lipid/mL) (●) or high (500 μg of neutral lipid/mL) (○) concentrations of [^{3}H]TGRP. Results are the mean ± SD of triplicate incubations.

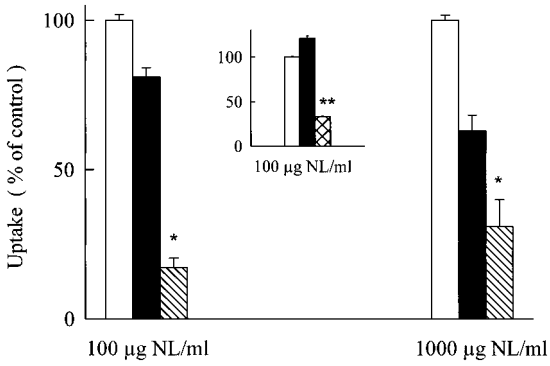

FIGURE 2: Effect of heparin on TGRP cell uptake in normal fibroblasts. Normal (receptor-positive) fibroblasts were incubated with low (100 μg of neutral lipid/mL) or high (1000 μg of neutral lipid/mL) particle concentrations with apo E (0.1 μg of apo E/μg of neutral lipid), in the absence (open bar) or presence of either 10 units/mL (solid bar) or 1000 units/mL (hatched bar) heparin. Results are expressed as the percent of the TGRP uptake measured in control cells. (Inset) Cells were incubated with 100 μg of neutral lipid/mL and apo E (0.1 μg of apo E/μg of neutral lipid) in the absence (open bar) or presence of heparin, 10 units/mL (solid bar), and in the presence of heparin (10 units/mL) plus a 2−3-fold molar excess of anti-apo E monoclonal antibody 1D7 (crosshatched bar). Results are expressed as the percent of the TGRP uptake measured in control cells. Results represent the mean ±SD of triplicate incubations (*P < 0.05, **P < 0.01 compared to control).

apo E at 25 °C with gentle agitation for 30 min, which allows sufficient time for equilibrium binding (Oswald & Quarford, 1987; Tajima et al., 1983). In our system it is likely that apo E is equally distributed among particles, since in a similar system with model emulsion particles, Tajima et al. (1983) have demonstrated that apo E distributes evenly among particles rather than concentrating on certain particles, leaving others with less apo E. Under the conditions used herein, all or almost all of the apo E present in the medium is bound to TGRP (Granot et al., 1994; Schwiegelshohn et al., 1995; Oswald & Quarford, 1987). The apo E−particle complex resulting from the preincubation was added to upregulated cells, and incubation was carried out at 37 °C on a rocker (Lab Line Instruments, Inc., Melrose Park, IL), for 4−8 h. After incubation, the cells were chilled on ice and washed twice with cold PBS containing 0.2% BSA and then washed twice with PBS alone. To measure particle uptake, cells were first treated with heparin (1400 units/mL of PBS) for 1 h at 4 °C. After heparin treatment, the cells were washed twice with PBS. When [^{3}H]cholesteryl ether was used for labeling, the cell-associated [^{3}H]cholesteryl ether was measured after lysis of the washed cells with 0.1 N NaOH. When [^{3}H]cholesteryl oleate was used, the [^{3}H]ester was extracted from the cells with hexane/2-propanol (3:2) and run on thin-layer chromatography (TLC). The solvent used was hexane/diethyl ether/acetic acid (70:30:1 v/v/v). Uptake calculated by determination of total (free and esterified) [^{3}H]-cholesterol gave values that were very similar to those observed with the [^{3}H]ether. In some experiments the emulsions contained ^{125}I-apo E, which was measured in NaOH cell lysates as described for [^{3}H]ether. The specific activities used were 1750−2750 cpm/μg of NL for [^{3}H] and 350−700 cpm/ng of apo E for ^{125}I. Based on radioactivity measurements, uptake was expressed as micrograms of NL or apo E per milligram of cell protein. Data are expressed as the mean ± SD of triplicate incubations.

Statistics. For the level of significance between experimental conditions, a two group paired two-tailed Student *t*-test was performed.

RESULTS

We assessed the effect of apo E concentration on particle uptake at different particle concentrations. Figure 1 shows uptake data for increasing amounts of apo E in normal fibroblasts. At a low particle concentration (25 μg of NL/mL), a 10-fold increase in apo E:particle ratio (from 0.01 μg of apo E/μg of NL to 0.10 μg of apo E/μg of NL) increased emulsion uptake by about 4−5-fold, compared to particles without apo E. At high particle concentration (500 μg of NL/mL), the corresponding increase in uptake was approximately 2-fold as apo E concentration increased. Thus, the presence of apo E enhances particle uptake at both low and high particle concentrations. Overall, increasing both particle concentration and apo E:particle ratios resulted in substantial increases in particle uptake.

We next initiated experiments to determine if multiple mechanisms contribute to apo E-mediated particle uptake at high particle concentrations. We examined the effects of heparin at different particle concentrations on uptake pathways in human skin fibroblasts, cells that do not express scavenger or VLDL apo E receptors (Figure 2). Low concentrations of heparin, such as 10 units/mL, can displace a number of ligands from cell surface HSPG (Ji et al., 1993; Brown et al., 1991; Choi et al., 1991), whereas high heparin concentrations, such as 1000 units/mL, will release apoprotein ligands from cell receptors (Ji et al., 1993; Brown et al., 1991; Choi et al., 1991). We thus studied the effects of low and high heparin concentrations on apo E−TGRP uptake.

Previous studies utilizing heparin as a tool in cell experiments focused largely on LDL and used high heparin concentrations (Goldstein et al., 1976). Since there is little available data on effects of low heparin concentration (∼10 units/mL) on uptake of apo E−TGRP particles, we designed experiments to address this. In fibroblasts, at low particle concentration (25 μg of NL/mL), where all or almost all uptake is via cell receptors, heparin concentration at 10 units/mL had no or very little effect on particle uptake. However, as heparin concentration increased from 50 to 1000 units/mL there was a progressive decrease of particle uptake to ≤15% of uptake in the absence of heparin (data not shown).

As shown in Figure 2, low-concentration heparin (10 units/mL) decreased particle uptake substantially at high, but not at low, particle concentrations (a nonsignificant <15% reduction using 100 μg of NL/mL versus 35% reduction using 1000 μg of NL/mL). High-concentration heparin (1000 units/mL) reduced particle uptake at high and low particle concentration (approximately 85% reduction) using 100 μg of NL/mL versus ~65% reduction using 1000 μg of NL/mL. At low particle concentration an anti-apo E monoclonal antibody 1D7, which inhibits LDL and LRP receptor-mediated uptake of apo E particles (Innerarity et al., 1983), resulted in about 65% inhibition of particle uptake; this was not affected by the presence of a low heparin concentration (10 units/mL), which had no effect on particle uptake (Figure 2, inset). (Control mouse IgG had no effects on particle uptake.) Similar effects were obtained studying apo E−particle uptake in J774 macrophages in the presence of heparin or anti-apo E antibody, 1D7 (data not shown). These results suggest that at low particle concentrations, most TGRP are bound to and internalized via the LDL receptor; however, at high concentrations, TGRP are likely bound to and internalized via other sites on the cell surface, different from classical lipoprotein receptor-mediated pathways.

To test whether HSPG was involved with particle binding and uptake at high particle concentrations, we studied apo E−TGRP uptake before and after removal of cell surface HSPG by heparinase/heparitinase (H/H) treatment in normal and LDL receptor-negative fibroblasts (Figure 3). In normal fibroblasts, at low particle concentration, removal of cell surface HSPG by H/H had little or no effect on particle uptake (Figure 3A). In contrast, at higher particle concentration (>100 μg of NL/mL), cell treatment with H/H reduced TGRP uptake to levels similar to saturation uptake via the LDL receptor pathway. Experiments with ^{125}I-apo E in which we measured apo E internalization and degradation gave similar results to those obtained with [^{3}H]cholesteryl ether (Figure 3A, inset). In fibroblasts that lack the LDL receptor, substantial uptake of apo E−TGRP only occurred at high particle concentrations (>250 μg/mL), and this uptake was almost completely abolished following treatment of cells with H/H (Figure 3B). Taken together, these results suggest that at low particle concentrations apo E−TGRP are internalized by a non-HSPG-dependent pathway via the LDL receptor. Since removal of HSPG does not affect particle uptake at low particle concentration, the affinity for binding and uptake of apo E−TGRP via the LDL receptor is higher than that of HSPG pathways. At high particle concentrations, however, a large fraction of particle uptake requires HSPG both in normal and LDL receptor-deficient cells. As well, in these cells the uptake capacity of the HSPG-dependent pathway is higher than that of the LDL receptor.

We next investigated if the amount of apo E per particle was important in determining the binding of apo E−TGRP to cell surface HSPG. At low particle concentration (25 μg of NL/mL) H/H did not appreciably alter particle uptake at any apo E/neutral lipid ratio (see below). However, at high particle concentration (500 μg of NL/mL) H/H treatment led to increasing effects as the apo E/neutral lipid ratio increased. With a ratio of 0.01 μg of apo E/μg of neutral lipid, H/H treatment decreased uptake by only 2%. However, at a ratio of 0.1 μg of apo E/μg of neutral lipid, 58% reduction was observed (data not shown). For the highest ratio used here (0.1 μg of apo E/μg of NL), we have estimated an average

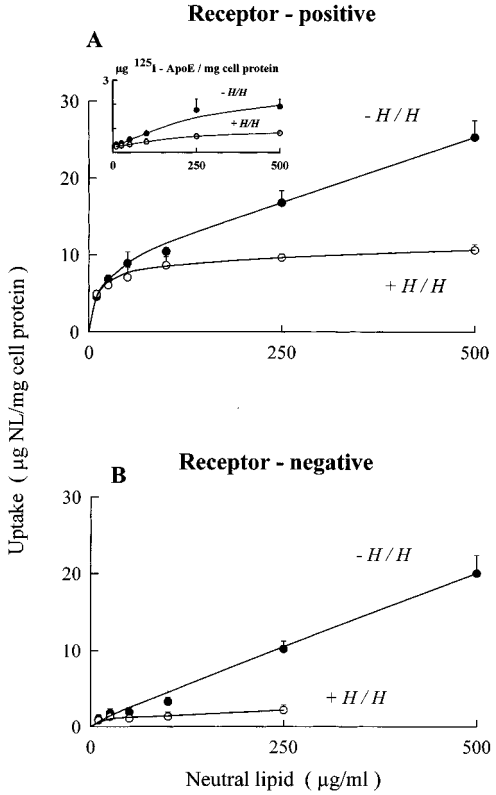

FIGURE 3: Effect of removal of cell surface HSPG on TGRP uptake in normal and LDL receptor-negative fibroblasts. (A) Normal (receptor-positive) fibroblasts were incubated at 37 °C for 4 h in media containing different concentrations of [^{3}H]TGRP and apo E (0.1 μg of apo E/μg of neutral lipid), in the absence (●) or presence (○) of heparinase/heparitinase (2.5 units each/mL). (Inset) Cells were incubated as described in the presence of different concentrations of [^{3}H]TGRP and ^{125}I-apo E. (B) Receptor-negative fibroblasts were treated as in panel A in the absence (●) or presence (○) of heparinase/heparitinase. All results are the mean ± SD of triplicate incubations.

of 4−6 apo E molecules/particle (Granot et al., 1994). These results suggest that when few apo E molecules are present per particle, apo E binds to high-affinity receptor sites on the cell surface and not appreciably to HSPG; when more apo E is present and receptors are saturated, binding to HSPG occurs.

It has been suggested that the LRP is another receptor that will play a role in uptake of apo E−TGRP, at least in liver cells. To assess the potential contribution of LRP-mediated uptake of apo E−TGRP, we studied the effects of the 39-kDa receptor-associated protein (RAP) at a concentration (10 μg/mL) that inhibits binding of a number of ligands to LRP (Warshawsky et al., 1994; Bu et al., 1992), and compared its effects to that of H/H. (In pilot studies not shown, higher concentrations of RAP did not demonstrate greater effects than RAP at 10 μg of NL/mL.) In normal fibroblasts, at low particle concentration (25 μg of NL/mL, 0.1 μg of apo E/μg of NL), neither RAP nor H/H treatment had any significant effect on TGRP uptake (Figure 4A). However, at high particle concentration (500 μg of NL/mL, 0.1 μg of apo E/μg of NL), RAP inhibited TGRP uptake by 20−25%. This inhibition was considerably less than that due to removal of cell surface HSPG by treatment with H/H; i.e., 50−60% decrease in particle uptake compared to nontreated cells. In receptor-negative fibroblasts, treatment with RAP had only minor effects on decreasing particle uptake at high concen-

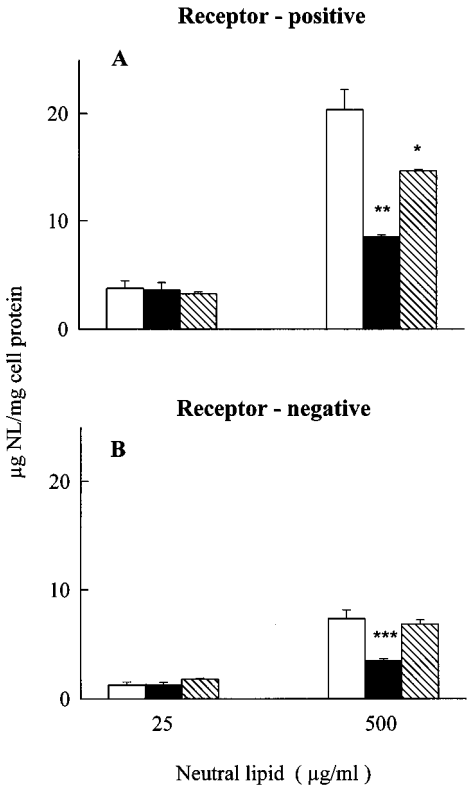

FIGURE 4: Effect of removal of cell surface HSPG and blocking of LRP on TGRP uptake by fibroblasts. (A) Receptor-positive and (B) receptor-negative fibroblasts were incubated at 37 °C for 4 h in media containing low (25 μg of neutral lipid/mL) or high (500 μg of neutral lipid/mL) concentrations of [^{3}H]TGRP (0.1 μg of apo E/μg of neutral lipid). Cell TGRP uptake was measured in the absence (open bars) or in the presence of either heparinase/heparitinase (2.5 units each/mL) to remove cell surface HSPG (solid bars) or RAP (10 μg/mL) to block the LRP (hatched bars). Results are the mean ± SD of triplicate incubations (*P < 0.05, **P < 0.01, ***P < 0.001 compared to control).

tration (500 μg NL/mL), but H/H removal of cell surface HSPG resulted in a major decrease in particle uptake (Figure 4B).

Inclusion of RAP in incubations with cells that had been H/H-treated led to no further decrease in TGRP uptake in either normal or receptor-negative cells (data not shown). These results demonstrate that when LRP contributes to TGRP uptake, it does so at particle concentrations greater than those required to saturate the LDL receptor. Moreover, as previously suggested by others, LRP-mediated uptake appears to depend upon the presence of HSPG on the cell surface (Beisiegel et al., 1994; Mahley et al., 1994). Finally, at physiologic particle concentrations, inhibition of LRP-mediated uptake by RAP is less than half that obtained after removal of HSPG. This indicates that a substantial number of particles can be internalized directly after binding to HSPG.

Of interest, in receptor-negative cells where most particle uptake is via HSPG, we noted in time course experiments that the rate of particle uptake was about half that in cells with the LDL receptor (data not shown). This is in keeping with our previous data (Rumsey et al., 1992) and that of others (Hamilton et al., 1990), which have shown the HSPG-mediated endocytosis pathways of a number of ligands are slower than uptake mediated by the LDL receptor.

We next questioned whether intracellular catabolism of TGRP would be substantially affected by different routes of

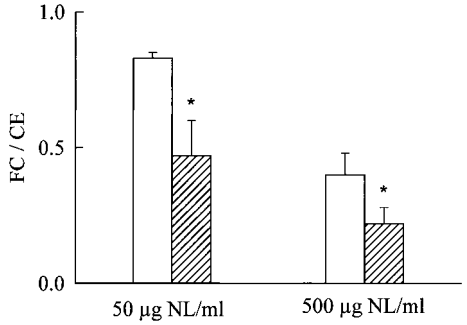

FIGURE 5: Intracellular partitioning of TGRP cholesteryl ester to free cholesterol vs cholesteryl ester. Normal receptor-positive (open bars) or receptor-negative fibroblasts (hatched bars) were incubated at 37 °C for 2 h in media containing low (50 μg of neutral lipid/mL) and high (500 μg of neutral lipid/mL) concentrations of TGRP containing ^{3}H-cholesteryl oleate with apo E (0.1 μg of apo E/μg of neutral lipid). Following extraction of the cells and thin-layer chromatography, the fraction of free cholesterol to cholesteryl ester was determined (FC/CE). Results are the mean ± SD of triplicate incubations (*P < 0.05 compared to receptor-positive cells).

delivery, i.e., via the LDL receptor-mediated pathway versus pathways associated with HSPG binding and internalization. To address this, we radiolabeled TGRP with [^{3}H]cholesteryl oleate rather than radiolabeled cholesteryl ether and compared the hydrolysis of cholesteryl oleate to free cholesterol in LDL receptor-negative and normal fibroblasts at low and high particle concentrations. Figure 5 shows that partitioning of particle cholesteryl ester between free cholesterol and cholesteryl ester is considerably higher after receptor-mediated internalization. These results suggest different intracellular metabolic consequences for TGRP internalized via HSPG as compared to LDL receptor-mediated pathways.

DISCUSSION

Our results show that in an extrahepatic cell line, human fibroblasts, apo E-containing TGRP are internalized by both LDL receptor-mediated and non-LDL receptor-mediated pathways. In these cells, at low particle concentration, TGRP uptake is mainly via the LDL receptor and is not appreciably affected by removal of cell surface HSPG. However, at particle concentrations approaching what might be found physiologically in plasma and interstitial fluid (neutral lipid concentrations of ≥250 μg/mL), apo E mediates particle uptake by binding to HSPG, which then leads to internalization of substantial amounts of TGRP. Our results demonstrate that HSPG binding does not enhance TGRP uptake via the high-affinity LDL receptor pathway. RAP, a ligand that competes for binding to LRP, is unable to block fully the uptake of apo E−TGRP. Thus, at higher particle concentrations, some particles are internalized directly through lower affinity but high capacity HSPG pathways involving neither LRP nor the LDL receptor. These mechanisms may be particularly important in extrahepatic tissues where, as suggested by Dietschy et al. (1993), nonreceptor mechanisms become increasingly important as lipoprotein concentrations rise.

After lipase-mediated hydrolysis of triglyceride in chylomicrons or VLDL, it has been suggested that the initial step in clearance of triglyceride-rich remnant particles from plasma is sequestration of these particles within the space of Disse, through an interaction with HSPG in the liver. Uptake of particles by hepatocytes would then be mediated by the LDL receptor and LRP. Ji et al. (1994) have

suggested that an apo E-enriched remnant lipoprotein, β-VLDL, binds avidly to HSPG and to LRP, but β-VLDL without the addition of excess apo E does not. There are a number of important differences between our approach and that of Ji et al. First, they used HepG2 cells, a human hepatoma cell line that secretes endogenous apo E. Our data were obtained using fibroblasts (and macrophages), cell lines that do not express or secrete apo E. Second, their studies were performed at relatively low concentrations of particles (1 μg of β-VLDL protein/mL). Further, to demonstrate effects with β-VLDL, addition of substantial amounts of exogenous apo E to β-VLDL was required. We found evidence of direct uptake via HSPG beginning only at higher particle concentrations. This suggests that HSPG-mediated uptake is important only at higher but more physiological particle concentrations (about 250 μg of NL/mL).

In view of the suggestion that apo E binding to receptors is multivalent, i.e., multiple apo E molecules on a single particle bind to multiple receptors, our results are similar to those of others in demonstrating that receptors are likely to become saturated only when there are at least 4−5 apo E molecules per particle (Ji et al., 1994). At this point, binding to HSPG begins to play a role. Our results, similar to those of Ji et al. (1994), indicate that H/H does not inhibit binding via the LDL receptor. In our experiments, addition of RAP had only a minor effect on diminishing TGRP uptake in receptor-negative cells either at low (25 μg of NL/mL) or high (500 μg of NL/mL) concentrations. In normal fibroblasts, an effect of RAP was observed only at high particle concentrations indicating that LRP contributes to apo E−particle uptake after the LDL receptor is saturated. While RAP can inhibit binding to the LDL receptor, its affinity for this receptor is much weaker than for LRP (Medh et al., 1995; Mokuno et al., 1994).

There are precedents for direct uptake of molecules via HSPG. Basic fibroblast growth factor (bFGF) is an example. Receptors provide a high-affinity binding site for this protein, while HSPG are a high-capacity but low-affinity bFGF cell surface binding site (Reiland & Rapraeger, 1993; Moscatelli, 1992). However, bFGF bound to either site is internalized and degraded. Similarly, previous data from our laboratory have suggested that lipoproteins bound to macrophage and fibroblast cell surfaces via lipoprotein lipase are internalized and degraded both via receptor and HSPG receptor-independent pathways (Obunike et al., 1994). However, similar to our results shown in Figure 5, degradation pathways are less efficient when particles are internalized via HSPG compared to the LDL receptor (Rumsey et al., 1992). Our results provide additional evidence for another HSPG-mediated secondary metabolic pathway that does not appear to require "classical" receptors. In our experiments, the VLDL (Takahashi et al., 1992) and the TGRP receptors (Ramprasad et al., 1995) are unlikely to represent appreciable routes of uptake, since they are only weakly expressed, if at all, by the cells used in our studies. Also, the receptor described by Ramprasad et al. (1995) does not recognize apo E.

While our studies were designed primarily to delineate the effects of apo E, we recognize that the lipid composition of the model TGRP emulsions used differs from that of native lipoproteins. Nevertheless, in a previous work we have shown that emulsions prepared from extracted human VLDL lipids behave, qualitatively and quantitatively, like pure lipid emulsions prepared in the laboratory (Granot et al., 1994). To avoid potentially confounding effects of other apoproteins, our particles contained only apo E to allow us to assess apo E binding directly. It is possible that other apoproteins will modulate the interaction of apo E−TGRP with HSPG, cell receptors, and other molecules on the cell surface. Also, most of our studies were done in fibroblasts and not hepatocyte-like lines. Unlike liver cells, fibroblasts do not secrete lipase, endogenous apo E, bile acids, or lipoprotein particles and thus offer a good model for apo E interactions with both LDL receptor and HSPG in the absence of other confounders.

While the relative amounts of receptors as compared to HSPG may be quite different in different cell lines, our experiments clearly delineate three distinct pathways whereby triglyceride-rich particles can be taken up by extrahepatic cells. In addition to uptake by the LDL receptor and LRP, we provide strong evidence for a third uptake mechanism; i.e., a nonreceptor pathway of direct uptake via HSPG. Two of the uptake pathways (LRP and HSPG) begin to function only after particle concentration increases above levels required to saturate the LDL receptor. We suggest that our results, based on experiments in nonhepatocyte cell lines, are likely relevant to uptake and metabolism of TGRP in peripheral extrahepatic tissues, e.g., bone marrow or the arterial wall, as compared to the overall clearance of lipoproteins from the plasma compartment, which largely occurs by liver-mediated removal (Hussain et al., 1989; Dietschy et al., 1993).

Are our results relevant to what occurs physiologically? Particle concentrations used in these studies are similar to those that exist in "portal" systems, such as bone marrow. Moreover, it is likely that concentrations of TGRP even higher than those used herein, may be reached in arterial tissues after damage to the vascular endothelium or to other tissues. Such a situation might even be exaggerated in the postprandial state when apo E−TGRP increase in plasma. Clearly apo E is a critical protein for clearance of TGRP from plasma, and variations in apo E structure will affect its binding not only to cell receptors but also to HSPG (Ji et al., 1994). TGRP and their remnants, which contain apo E, may contribute to the atherosclerotic process (Zhang et al., 1992). The absence of apo E in human subjects or in apo E knockout mice is also associated with premature atherosclerosis (Plump et al., 1992; Zhang et al., 1992). In both human and animal apo E deficiency states, TGRP plasma levels are markedly elevated, and these particles almost certainly enter cells largely through nonreceptor pathways. However, since LDL receptor defects alone are generally not associated with marked changes in clearance of TGRP or remnants (Willnow et al., 1994; Ji et al., 1994), it is likely that other pathways play an important role. In vivo evidence of the importance of such pathways for chylomicron remnant removal were recently described in mutant mice lacking the LDL receptor (Herz et al., 1995). Using mice homogenous for both apo E and LDL receptor deficiency, Fazio et al. (1996) reported preliminary data indicating that LRP cannot account for all apo E-enriched TGRP clearance in the absence of the LDL receptor. Our data suggest that in addition to clearance pathways of TGRP via the LDL receptor, apo E interactions with HSPG will promote TGRP clearance by LRP-dependent and independent mechanisms. Moreover, HSPG-dependent clearance likely contributes increasingly to particle removal

once TGRP concentrations rise past levels that saturate LDL receptors.

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