Multiple processes are involved in the uptake of chylomicron remnants by mouse peritoneal macrophages

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Abstract **The processes responsible for the uptake of chylomicron remnants by macrophages were investigated using freshly isolated cells from low density lipoprotein (LDL) receptor, very low density lipoprotein (VLDL) receptor and apolipoprotein E knockout mice. In peritoneal macrophages from normal mice, the metabolism of chylomicron remnants was inhibited 40% by anti-LDL receptor antibody and 60% by a high concentration of receptor-associated protein (RAP). Together they reduced the amount processed by 70%. Digestion of cell proteoglycans decreased remnant degradation by 20% while the addition of acetyl-LDL had no effect. When LDL receptors were absent, the absolute rates of metabolism were less than that of normal cells and were not inhibited by the anti-LDL receptor antibody; the rates, however, were reduced to less than half by RAP. These suggest that the LDL receptor-related protein (LRP) or another LDL receptor family member(s) contributes to chylomicron remnant uptake and becomes the major mechanism of uptake when LDL receptors are absent. In contrast, the VLDL receptor was not involved as its absence did not affect chylomicron remnant metabolism. Similarly, the absence of apoE production did not affect the amount of remnant uptake; however, the proportion that was sensitive to RAP was eliminated. The level of LRP expression was not altered in these cells whereas there was a decrease in LDL receptors. This suggests that the apoE content of chylomicron remnants is sufficient for its recognition by LDL receptors but additional apoE is required for its uptake by the LRP and that there is an up-regulation of a non-LDL receptor family mechanism in apoE deficiency. Together these studies suggest that even in the absence of LDL receptors or apoE secretion, chylomicron remnants could contribute to lipid accumulation in the artery wall during atherogenesis.—**Fujioka, Y., A. D. Cooper, and L. G. Fong. **Multiple processes are involved in the uptake of chylomicron remnants by mouse peritoneal macrophages.** ***J. Lipid Res.*** **1998.** 39: **2339–2349.**

Supplementary key words apoE • VLDL receptor • LDL receptor • knockout mice • THP-1 cells • LRP • RAP • scavenger receptor

Chylomicron remnants are formed after the hydrolysis of triglycerides, from intestine-derived chylomicrons, by lipoprotein lipase (LPL) and are rapidly removed from the circulation. The principal tissue site of removal is the liver (1, 2). The mechanisms responsible for this rapid clearance are becoming more defined. Several mechanisms that appear to play a major role under normal circumstances are the low density lipoprotein (LDL) receptor (3, 4), the LDL receptor-related protein (LRP) (5–7), and the localized production of apolipoprotein E (apoE) (8–10). The delay of clearance of these triglyceride-rich lipoprotein particles induced by any of several genetic or metabolic disorders is characterized clinically as a mixed hyperlipemia and is often associated with accelerated atherosclerosis. It has been suggested that chylomicron remnants are direct initiators of atherosclerosis (11–13). This concept is supported by several carefully conducted studies that have correlated a delay in remnant clearance with an increased risk of developing atherosclerotic cardiovascular disease (14–18).

Macrophages are thought to play an important role in the formation of the atherosclerotic lesion, particularly the fatty streak, the earliest identifiable lesion. This is characterized by the presence of lipid-laden cells termed foam cells, a majority of which are derived from macrophages (19–21). The precise process that accounts for the generation of macrophage-derived foam cells is unknown. One pathway, currently supported by substantial evidence, involves oxidation of LDL followed by removal of this by receptors on macrophages (22). This, however, would not explain atherogenesis in instances of low LDL levels. In such cases, delayed remnant removal is often present. Prolonged elevation of levels of chylomicron remnants in the

Abbreviations: LPL, lipoprotein lipase; LDL, low density lipoprotein; LRP, LDL receptor-related protein; VLDL, very low density lipoproteins; RAP, receptor-associated protein; FBS, fetal bovine serum; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; LPDS, lipoprotein-deficient serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; β-VLDL, β-very low density lipoprotein.

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circulation could promote their entry into the artery wall and contribute to lesion formation by modulating cellular activities or by serving directly as a source of lipoprotein cholesterol. In general, macrophages appear to be a tissue site of uptake of remnants and, with delayed removal by liver, increased macrophage uptake can be expected and has been reported (23–25). Monocytes and macrophages have several pathways that could mediate chylomicron remnant uptake. These include the LDL receptor, other members of the LDL receptor gene family, including the LRP and the very low density lipoprotein (VLDL) receptor (26), the scavenger receptor family, and the human triglyceride-rich lipoprotein receptor (27, 28). Our group and another previously reported that the uptake of chylomicron remnants by normal macrophages is mediated primarily by the LDL receptor (25, 29, 30). These studies were carried out primarily in macrophage-derived cell lines. In primary macrophages, it was noted that another pathway(s) may have been involved to a greater relative degree. Chylomicron remnants are enriched with apoE, an apolipoprotein that binds with high affinity to LDL receptor, the LRP, and the VLDL receptor (31). In addition, macrophages can produce apoE and LPL (32–34), which could enhance the binding of chylomicron remnants by macrophages and accelerate chylomicron remnant uptake and metabolism. To begin to delineate the relative contributions of the different receptor-dependent mechanisms, we studied the metabolism of chylomicron remnants using two complementary approaches. First, the effects of inhibition of the different receptor mechanisms using the receptor-associated protein (RAP) and an LDL receptor blocking antibody were determined. Second, the metabolism of chylomicron remnants by macrophages harvested from mice that do not express the LDL receptor, VLDL receptor, or produce apoE were determined. From these studies, we conclude that several pathways are involved in the uptake of chylomicron remnants by macrophages, and their relative contributions depend on the functional status of the macrophages.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and fetal bovine serum (FBS) were purchased from GIBCO Laboratories (Grand Island, NY). Phorbol 12-myristate 13-acetate (PMA) was obtained from LC Laboratories (Woburn, MA). This was dissolved in DMSO and stored at −20°C until use. Heparinase III and chondroitinase ABC were obtained from Sigma (St. Louis, MO). ^{125}I-Na (carrier-free) and the ECL Western blotting analysis system were purchased from Amersham Corp. (Arlington Heights, IL). Bio-Gel P-6 packed column (Econo-Pac 10DG) and Bio-Gel A-50m (100–200 mesh) were purchased from Bio-Rad (Richmond, CA). A plasmid containing the cDNA for RAP fused with glutathione-S-transferase was kindly provided by Dr. D. Strickland (35). The protein was purified in our laboratory as described earlier (36). The rabbit polyclonal anti-LDL receptor and anti-LRP antibodies used have been previously described and characterized elsewhere (25, 37, 38). Nonimmune IgG was isolated from normal rabbit sera using the same procedure.

Animals

Normal control mice (Swiss Webster or B6,129F2 control), the LDL receptor knock-out mice (B6,129-Ldlr^{tmlHer}), and the VLDL receptor knock-out mice (B6,129-Vldlr^{tmlHer}) were purchased from Jackson Laboratory (Bar Harbor, ME). The apoE knock-out mice were a gift from Dr. E. Rubin at the University of California, Berkeley (39). Male Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA) for the preparation of chylomicrons and chylomicron remnants. The animals had free access to food and water.

Cells

Resident or thioglycollate-elicited mouse peritoneal macrophages were isolated as described previously (25, 40, 41). Cells were added to 24-well tissue culture plates (Nunc; Roskilde, Denmark) or 60-, 100-mm tissue culture dishes (Falcon; Becton Dickinson, Oxnard, CA) in DMEM supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and l-glutamine (292 μg/ml) (medium A) and incubated for 1 h under a humidified atmosphere of 95% air and 5% CO_2. The adherent cells were used in lipoprotein uptake assays or for the preparation of cell membranes. THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and l-glutamine (292 μg/ml) (medium B). The cells were seeded $5.0 \times 10^6/cm^2$ in 24-well plates in medium B supplemented with 0.1 μm PMA and 10% bovine lipoprotein-deficient serum instead of 10% FBS. The cells were used in uptake assays after incubation for 48 h.

Lipoproteins

Rat mesenteric lymph chylomicrons and chylomicron remnants were prepared in vivo as previously described (25, 42). Selected preparations of chylomicron remnants were analyzed to verify their protein and lipid compositions. The relative protein values were 13% apoprotein (apo) B-48, 3% albumin, 12% apoA-IV, 52% apoE, and 20% of an unidentified protein of 120 kDa. The triacylglycerol content was 15.4 ± 1.0 per mg protein and cholesterol content was 3.8 ± 0.3 per mg protein (n = 13). The chylomicron remnants were iodinated by a modification of the iodine monochloride methods (25). Human LDL, acetylated LDL, and oxidized LDL were prepared by established methods (41, 43, 44).

Lipoprotein degradation and association studies

Cell degradation and association of lipoproteins were measured as described previously (40, 45). The incubation medium was DMEM (mouse macrophages) or RPMI 1640 (THP-1 cells) supplemented with 20 mm HEPES and 0.5% BSA (pH 7.4). The cells were incubated with ^{125}I-labeled chylomicron remnants (0.5 μg/ml) in the absence or presence of unlabeled chylomicron remnants, anti-LDL receptor antibody, RAP, or a combination of these in amounts described in the text. After incubation at 37°C for 4 h, the medium was removed and the cell monolayers were washed three times with PBS. The amount of degradation products in the medium was measured after trichloroacetic acid (TCA) and silver nitrate precipitation. The cells were then dissolved in 0.1 N NaOH for the determination of cell association and protein content. The amount of specific metabolism was calculated by subtracting the amount of nonspecific metabolism (measured in the presence of 10 μg/ml unlabeled chylomicron remnants) from the total amount processed (measured in the absence of unlabeled chylomicron remnants). The effects of the anti-LDL receptor antibody and/or RAP on the specific metabolism of chylomicron remnants were then calculated. All values were also corrected for lipoprotein incubated in the absence of cells and expressed in ng/mg cell protein. Unless otherwise indicated, the means ± standard errors of a minimum of three different experiments are shown.

Digestion of cells with heparinase and chondroitinase

The digestion of macrophage proteoglycans was conducted as described by Kaplan and coworkers (46). Adherent elicited macrophages were incubated with 0.4 U/ml of heparinase III and chondroitinase ABC in DMEM containing 0.2% BSA at 37°C for 1 h. The specific metabolism of chylomicron remnants was then measured as described above except the enzymes were present throughout the incubation.

Preparation of solubilized cell extracts

Triton X-100 detergent-soluble cell extracts were prepared as described previously (45) and used for immunoblotting and ligand blotting studies. Mouse macrophages were cultured in 60- or 100-mm dishes per group instead of 24-well dishes to obtain sufficient amounts of cells. The cells were washed three times with ice-cold PBS and scraped into 1 ml of lysis solution [1% Triton X-100, 50 mm Tris (pH 8.0 at 4°C), 100 mm KCl, 1 mm phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 50 μm leupeptin] and incubated for 10 min on ice. The cell lysate was centrifuged (10,000 *g*; 10 min at 4°C) and the supernatant was stored at −80°C until analysis. In some experiments, cells were pretreated with pronase (0.5 mg/ml) at 4°C for 2 h to digest surface proteins (45). The enzyme-containing solution was removed and ice-cold medium A was added to quench remaining enzyme. The cells were washed 3 times with ice-cold PBS and cell extracts were prepared as described above.

Immunoblotting and ligand blotting of cell extracts

Aliquots of the detergent-soluble cell extracts (200–300 μg of protein) were applied to 3–15% gradient SDS-polyacrylamide gels under nonreducing conditions (37). The proteins were transferred to nitrocellulose paper by electroblotting. The section of nitrocellulose corresponding to the lane containing the molecular weight standards was cut off and stained with Amido Black [1% (w/v) in 45% ethanol and 10% acetic acid] and destained in water. The remainder was processed for immunoblotting or ligand blotting (25). Detection of the LDL receptor or LRP was performed using polyclonal rabbit antibodies as described previously (25) except that the immune complexes were visualized by enhanced chemiluminescence (ECL; Amersham). The bands were scanned by laser densitometry and the relative levels were quantified using Image Quant software (Molecular Dynamics). Ligand blotting was carried out as described previously (25) with ^{125}I-labeled chylomicron remnants (1 μg/ml). Bound chylomicron remnants were detected by autoradiography and the amount of binding were measured by laser densitometry.

Other assays

Protein was measured by the method of Lowry et al. (47) or that described for the Pierce BCA protein assay method (Pierce; Rockford, IL) using BSA as a standard.

Statistics

Statistical analysis was done using non-paired Student's *t*-test.

RESULTS

Degradation and cell association of chylomicron remnants by macrophages from normal mice

In our previous studies of chylomicron remnant uptake by macrophages and macrophage-like cells, we noted that, in the latter, the large preponderance of remnant uptake was by the LDL receptor (25, 29, 30); however, uptake by primary cells was lower and was incompletely inhibited by anti-LDL receptor antibody. Accordingly, the pathway(s) for the uptake of chylomicron remnants by macrophages was re-examined, initially using resident peritoneal macrophages from normal mice. In agreement with earlier studies, the degradation and cell association of chylomicron remnants by these cells was specific and saturable, and reduced by 70% when a 20-fold excess of unlabeled chylomicron remnants was added (not shown). Larger amounts of unlabeled chylomicron remnants (>10 μg/ml) often caused cell toxicity as determined by a loss of adherent cells. Thus, this concentration of unlabeled chylomicron remnants (10 μg/ml) was used for all subsequent studies to measure specific cell degradation and association. This may, however, result in an underestimate of the specific association and degradation.

To measure the proportion of the specific uptake that was mediated by the LDL receptor, an antibody that blocks ligand binding was included in the incubation. This antibody does not bind to the LRP or the VLDL receptor (48) but completely inhibits the specific cell association and degradation of LDL by mouse macrophages (not shown). The antibody suppressed chylomicron remnant uptake in a concentration-dependent manner with a maximum inhibition at 200 μg/ml. At this concentration, chylomicron remnant degradation and cell association were reduced 31.8% and 52.0%, respectively, when compared to the non-immune IgG control group (**Fig. 1**). Total uptake (degradation + cell association) was suppressed by 46.2% ± 4.2 (mean ± SE; n = 4). In subsequent studies, 200 μg/ml of polyclonal anti-LDL receptor antibody and non-immune IgG was used.

The RAP blocks the binding of all known ligands to the LRP and the VLDL receptor at low concentrations (35, 49–51) and at high concentrations, it also inhibits binding to the LDL receptor (49). RAP reduced specific chylomicron remnant uptake by resident macrophages in a con-

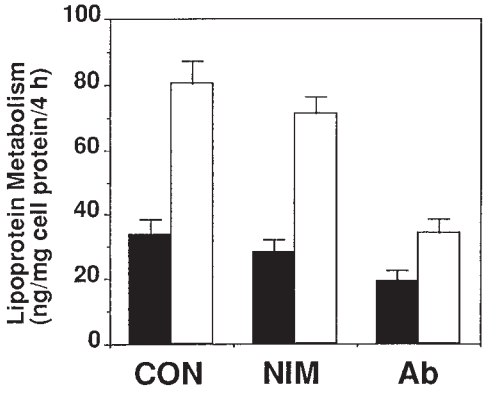

Fig. 1. Inhibition of chylomicron remnant metabolism by anti-LDL receptor antibody. Macrophages from normal mice were incubated with ^{125}I-labeled chylomicron remnants in the absence (CON) and presence of 200 μg/ml non-immune IgG (NIM) or anti-LDL receptor antibody (Ab) at 37°C for 4 h and the degradation (filled bars) and cell association (open bars) were measured as described in Methods. The results are expressed as the amount of specific metabolism by cells in ng/mg cell protein. Mean ± SE (n = 4 experiments) is shown.

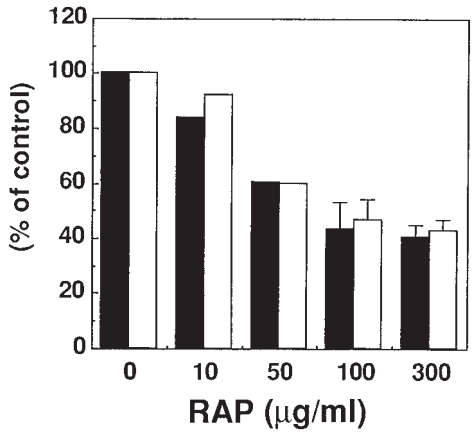

Fig. 2. Inhibition of chylomicron remnant metabolism by RAP. Macrophages from normal mice were incubated with ^{125}I-labeled chylomicron remnants in the presence and absence of RAP at the concentrations shown at 37°C for 4 h and the degradation (filled bars) and cell association (open bars) were measured as described in Methods. The results are expressed as a percentage of the specific metabolism by cells incubated with no addition. The average specific degradation and cell association for control resident cells were 31.0 ± 3.9 and 70.2 ± 4.8 ng/mg cell protein, respectively. Mean ± SE (n = 4 experiments) is shown.

centration-dependent manner (**Fig. 2**). A maximal effect of 60% was achieved at a concentration of 300 μg/ml. This was compatible with an amount found in previous studies to block almost all of the binding to the members of the LDL receptor family. Consistent with this was the inhibition of specific LDL uptake by mouse macrophages at 100 μg/ml RAP (96.2% ± 2.9 inhibition; n = 3). In order to dissociate the effects of RAP on the LRP from that on the LDL receptor, the effect of RAP (300 μg/ml) combined with the anti-LDL receptor antibody (200 μg/ml) was tested. The two combined inhibited specific chylomicron remnant degradation and association to 30% of control, although this was not significantly greater than that produced with RAP alone. The same experiments were carried out with thioglycollate-elicited macrophages. The absolute rates of chylomicron remnant uptake and the proportion inhibited by the anti-LDL receptor antibody, RAP, and the two combined were similar to that in resident cells (not shown).

Previous studies have shown that unlabeled acetyl-LDL was unable to inhibit the association of chylomicron remnants with J774 cells, a mouse macrophage cell line (25). To determine whether the scavenger receptor and its related receptor members contribute to remnant metabolism in primary mouse macrophages, the effects of acetyl-LDL (15 μg/ml) and fucoidan (100 μg/ml) were tested. Neither affected the degradation or association of chylomicron remnants (not shown).

Cell surface heparan sulfate proteoglycans (HSPG) have been suggested to participate in the metabolism of lipoproteins by modulating their association and uptake by cells (46, 50, 52–54). To begin to determine whether HSPG participate in the metabolism of chylomicron remnants by macrophages, elicited cells were pretreated with the enzymes heparinase III and chondroitinase ABC to digest cellular HSPG (46) and its effect on lipoprotein uptake was measured. The specific degradation was reduced from 34.0 ± 3.2 ng/mg protein to 26.7 ± 2.5 ng/mg protein (n = 3). This suggests that cell-associated proteoglycans contribute to the metabolism of chylomicron remnants by macrophages.

From these data, it can be concluded that at least 40% of chylomicron remnant uptake by resident macrophages is due to the LDL receptor and 20% due to the other members of the LDL receptor family. Together, the LDL receptor family members account for at least 70% of uptake. Because of the toxicity at high remnant concentrations, these may be underestimates; however, the possibility that a non-LDL receptor family member, non-scavenger receptor, makes a small contribution to normal uptake remained. Accordingly, we initiated studies in cells obtained from mice with genetic abnormalities of the lipoprotein receptor systems.

Degradation and cell association of chylomicron remnants by macrophages from LDL receptor knockout mice

To further quantify the role of the LDL receptor in chylomicron remnant metabolism, and to begin to investigate whether chylomicron remnants can contribute to lipid deposition and foam cell formation in the absence of functional LDL receptors, the metabolism of chylomicron remnants by resident and elicited macrophages from LDL receptor knockout mice was studied. There was no difference between resident and elicited macrophages (not shown), thus only the results for elicited macrophages is reported (**Fig. 3**). There was measurable specific uptake in these cells, however, the absolute rate of metabolism was less than that of macrophages from normal mice (see

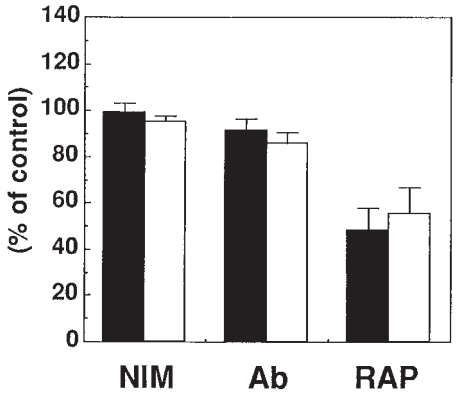

Fig. 3. Metabolism of chylomicron remnants by macrophages from LDL receptor knockout mice. Thioglycollate-elicited macrophages from LDL receptor knockout mice were incubated with ^{125}I-labeled chylomicron remnants in the presence and absence of 200 μg/ml non-immune IgG (NIM), 200 μg/ml anti-LDL receptor antibody (Ab), or 300 μg/ml RAP (RAP) at 37°C for 4 h and the degradation (filled bars) and cell association (open bars) were measured as described in Methods. The results are expressed as a percentage of the specific metabolism by cells incubated with no addition. The average specific degradation and cell association for control elicited cells were 9.7 ± 1.6 and 35.4 ± 3.5 ng/mg cell protein, respectively. Mean ± SE (n = 3 experiments) is shown.

below). This is consistent with the LDL receptor being a mechanism of quantitative importance. As expected, degradation and association of chylomicron remnants were not affected by the anti-LDL receptor antibody in these cells. Degradation and association were, however, reduced to less than half by RAP. Combining the anti-LDL receptor antibody with RAP had no additional effect (not shown). These data demonstrate that when LDL receptors are absent or down-regulated, macrophages can metabolize chylomicron remnants by another member(s) of the LDL receptor family. The absolute amount mediated by the other member(s) of LDL receptor family is consistent with the estimates of the studies with macrophages from normal mice and the possibility of a small amount of uptake mediated by a non-LDL receptor gene family member still remains.

Degradation and cell association of chylomicron remnants by macrophages from VLDL receptor knockout mice

The VLDL receptor is a member of the LDL receptor gene family and binds apoE-containing lipoproteins with high affinity. It is sensitive to RAP at relatively low concentrations (51). To estimate the role of the VLDL receptor in chylomicron remnant uptake by macrophages, cells from VLDL receptor knockout mice were studied (**Fig. 4**). In elicited macrophages from VLDL receptor knockout mice, there was specific uptake of chylomicron remnants. The absolute rate of chylomicron remnant metabolism was similar to that of macrophages from normal mice (see below). The anti-LDL receptor antibody inhibited chylomicron remnant uptake by 40% and a slightly greater inhibition was produced when RAP was added alone. When RAP combined with the anti-LDL receptor antibody were tested, the extent of the inhibition was not statistically different from that of RAP alone (not shown). On average, the metabolism of chylomicron remnants by macrophages from normal and VLDL receptor knockout mice was not different. This suggests that the role of the VLDL receptor is quantitatively minor in the uptake of chylomicron remnants or is replaced by a compensatory mechanism.

Degradation and cell association of chylomicron remnants by macrophages from apoE knockout mice

To learn whether additional apoE secretion is required for uptake by some of the LDL receptor family members in macrophages, as it is in other cell types (7, 50), cells from apoE knockout mice were studied (**Fig. 5**). In elicited macrophages from these mice, there was specific uptake of chylomicron remnants. These remnants contained the apoE of the rats in which they were prepared. The amount of specific uptake of chylomicron remnants by these cells was similar to that by macrophages from normal mice (**Fig. 6**). As was observed with macrophages from normal mice, the anti-LDL receptor antibody inhibited chylomicron remnant uptake by 40%. In contrast to the normal cell type, the addition of RAP was not able to inhibit chylomicron remnant uptake to a greater degree than by the anti-LDL receptor antibody. RAP combined with the anti-LDL receptor antibody did not significantly increase inhibition from that of RAP or the anti-LDL receptor antibody alone (not shown). This suggests that in the cell association and degradation of chylomicron remnants, the LDL receptor does not require the secretion of additional apoE, but uptake that can be attributed to other members of the LDL receptor family does require the secretion of additional apoE by the macrophages.

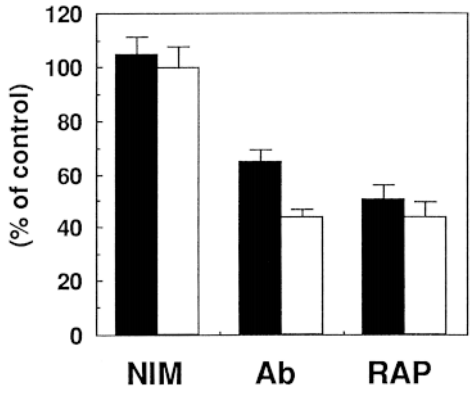

Fig. 4. Metabolism of chylomicron remnants by macrophages from VLDL receptor knockout mice. Thioglycollate-elicited macrophages from VLDL receptor knockout mice were incubated with ^{125}I-labeled chylomicron remnants in the presence and absence of 200 μg/ml non-immune IgG (NIM), 200 μg/ml anti-LDL receptor antibody (Ab), or 300 μg/ml RAP (RAP) at 37°C for 4 h and the degradation (filled bars) and cell association (open bars) were measured as described in Methods. The results are expressed as a percentage of the specific metabolism by cells incubated with no addition. The average specific degradation and cell association for control elicited cells were 33.6 ± 4.1 and 86.6 ± 2.1 ng/mg cell protein, respectively. Mean ± SE (n = 4 experiments) is shown.

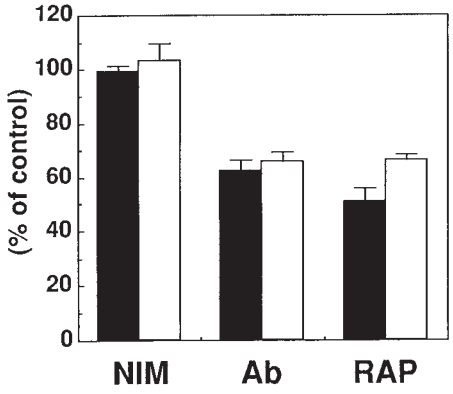

Fig. 5. Metabolism of chylomicron remnants by macrophages from apoE knockout mice. Thioglycollate-elicited macrophages from apoE knockout mice were incubated with ^{125}I-labeled chylomicron remnants in the presence and absence of 200 μg/ml non-immune IgG (NIM), 200 μg/ml anti-LDL receptor antibody (Ab), or 300 μg/ml RAP (RAP) at 37°C for 4 h and the degradation (filled bars) and cell association (open bars) were measured as described in Methods. The results are expressed as a percentage of the specific metabolism by cells incubated with no addition. The average specific degradation and cell association for control elicited cells were 20.1 ± 4.1 and 61.2 ± 14.8 ng/mg cell protein, respectively. Mean ± SE (n = 3 experiments) is shown. The amount metabolized between the anti-LDL receptor antibody and RAP were not statistically different.

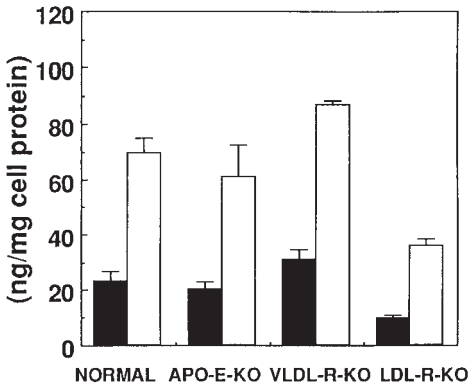

Fig. 6. Comparison of the absolute rates of chylomicron remnant metabolism by macrophages from normal, apoE knockout, VLDL receptor knockout, and LDL receptor knockout mice. Thioglycollate-elicited macrophages from normal, apoE knockout, VLDL receptor knockout, and LDL receptor knockout mice were incubated with ^{125}I-labeled chylomicron remnants at 37°C for 4 h and the degradation (filled bars) and cell association (open bars) were measured as described in Methods. The results are expressed as the amount of specific metabolism in ng/mg cell protein. Mean ± SE is shown.

Comparison of the absolute rates of degradation and cell association of chylomicron remnants by macrophages from normal, LDL receptor knockout, apoE knockout, and VLDL receptor knockout mice

The absolute amounts of degradation and association of chylomicron remnants by macrophages from the four strains of mice were also compared. Consistent with the loss of LDL receptors, chylomicron remnant degradation and association by elicited macrophages from LDL receptor knockout mice was reduced to almost 50% of that of normal mice (Fig. 6). This was consistent with the estimate that the LDL receptor was responsible for at least 40% of chylomicron remnant removal. However, the results with the apoE knockout mice were surprising. There was no reduction in degradation and association of chylomicron remnants by macrophages. Given the requirement of the LRP for additional apoE secretion, it is unlikely that this receptor was contributing to this compensation. This suggests that, in this animal, where atherogenesis is markedly accelerated, the LDL receptor or a non-LDL receptor family mediated mechanism for chylomicron remnant uptake by macrophages is induced. There was no difference in degradation and association of chylomicron remnants between elicited macrophages from normal and VLDL receptor knockout mice. This suggests that the VLDL receptor, one member of the LDL receptor gene family, has a minor role, if any, for chylomicron remnant uptake by macrophages (55).

Consistent with the metabolic studies was the extent of lipid accumulation by elicited macrophages from normal, LDL receptor knockout, and apoE knockout mice. The three types of cells were incubated with chylomicron remnants (10 μg/ml) for 48 h and the accumulation of lipid was visualized by oil-red-O staining. In macrophages from normal mice, there were distinct lipid droplets in the cytoplasm that were stained with oil-red-O (**Fig. 7**). These were not present in macrophages incubated in medium alone. Definite staining was also observed in cells from both the LDL receptor and apoE knockout mice but the staining appeared more diffuse. These studies demonstrate that chylomicron remnants can lead to extensive lipid accumulation in normal macrophages and that remnants can promote lipid deposition even in the absence of the LDL receptor or apoE production.

Comparison of LDL receptor and LRP expression in macrophages from normal, LDL receptor knockout mice, and apoE knockout mice

To determine whether the metabolism of chylomicron remnants by macrophages that do not produce apoE or express functional LDL receptors is due partly to an up-regulation of the LDL receptor or LRP, respectively, the

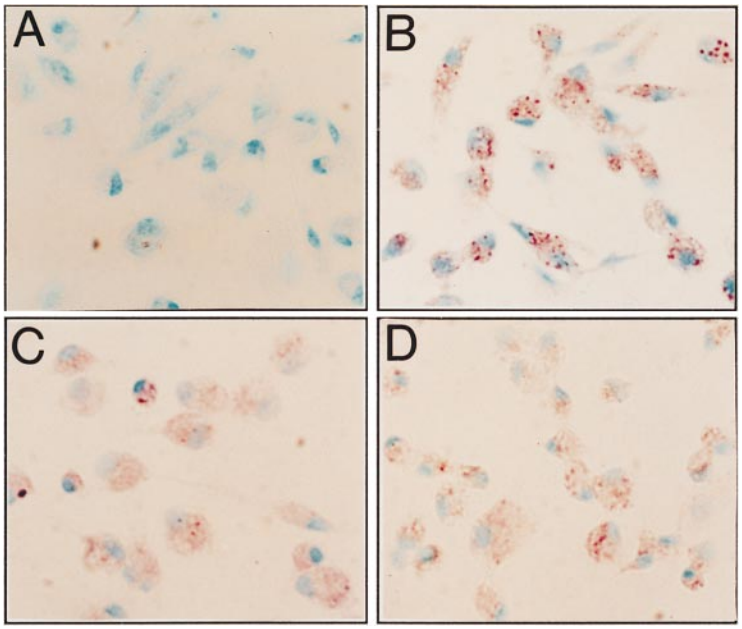

Fig. 7. Comparison of the accumulation of cellular lipid by macrophages from normal, apoE knockout, and LDL receptor knockout mice. Thioglycollate-elicited macrophages from normal, LDL receptor knockout, and apoE knockout mice were incubated with chylomicron remnants (10 μg/ml) in DMEM supplemented with 10% LPDS at 37°C for 48 h. The cells were fixed and stained with oil-red-O and counter-stained with methyl green. Panel A: normal macrophages alone; panel B: normal macrophages plus chylomicron remnants; panel C: macrophages from LDL receptor knockout mice plus chylomicron remnants; panel D: macrophages from apoE knockout mice plus chylomicron remnants. Macrophages from LDL receptor knockout and apoE knockout mice that were incubated without chylomicron remnants appeared identical to normal macrophages incubated without chylomicron remnants (not shown).

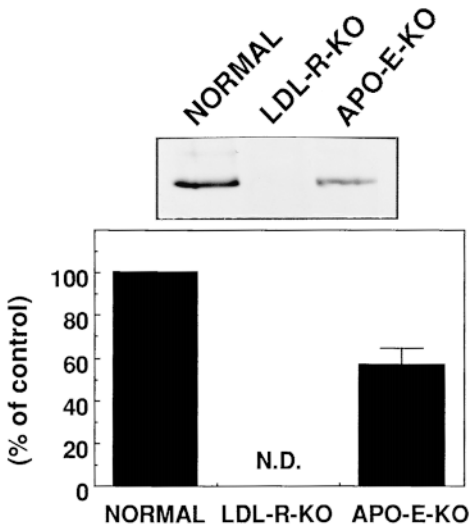

Fig. 8. LDL receptor expression by macrophages from normal, LDL receptor knockout, and apoE knockout mice. Thioglycollate-elicited macrophages were plated in 100-mm dishes and cellular extracts were dissolved with Triton X-100 prepared as described in Methods. Equivalent amounts of protein (200 μg) were separated by 3–15% gradient SDS-PAGE, transferred to nitrocellulose paper, and immunoblotted using anti-LDL receptor antibody. The immune complexes were detected by ECL and the bands were scanned by laser densitometry. Upper panel: a representative immunoblot that compares the level of LDL receptor expression by macrophages from normal, LDL receptor knockout (LDL-R-KO), and apoE knockout (APO-E-KO) mice. Lower panel: LDL receptor expression tabulated as a percentage relative to the density of that of macrophages from normal mice. The mean ± SE (n = 3 experiments) is shown. N.D., not detectable.

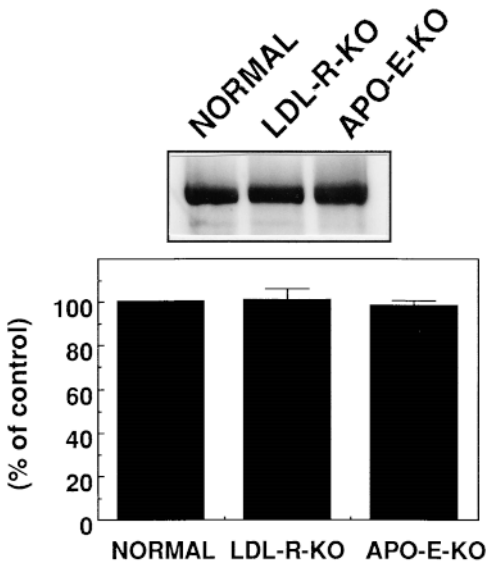

Fig. 9. LRP expression by macrophages from normal, LDL receptor knockout, and apoE knockout mice. Macrophage cell extracts were prepared from normal, LDL receptor knockout, and apoE knockout mice and equivalent amounts of protein (300 μg) were analyzed as described in the legend to Fig. 8. Upper panel: a representative immunoblot that compares the level of LRP expression by macrophages from normal, LDL receptor knockout (LDL-R-KO), and apoE knockout (APO-E-KO) mice. Lower panel: LRP expression tabulated as a percentage relative to the density of that of normal macrophages. The mean ± SE (n = 3 experiments) is shown.

level of expression of the LDL receptor and the LRP in macrophages from normal, LDL receptor knockout, and apoE knockout mice was compared by immunoblotting using antibodies specific for the LDL receptor and the LRP (**Fig. 8**). In the upper panel, a representative immunoblot that compares the level of expression of the LDL receptor by the three cell types is shown. In the lower panel, the level of receptor expression in the knockout mice relative to that in macrophages of normal mice in three different experiments is plotted. As expected, the LDL receptor was absent in the cells from the LDL receptor knockout mice. Interestingly, LDL receptor levels were reduced by 60% in the cells of apoE knockout mice. This was observed consistently. In contrast, there was no alteration in the level of the LRP (**Fig. 9**). This suggests that in the presence of hyperlipidemia in the apoE-deficient mice, macrophages accumulate cholesterol to a greater degree than macrophages from normal animals and this leads to down-regulation of the LDL receptor.

Comparison of ligand blotting of chylomicron remnants in macrophages from normal, LDL receptor knockout mice, and apoE knockout mice

The reduced number of LDL receptors in macrophages from apoE knockout mice but a normal rate of chylomicron remnant metabolism suggests the contribution of an alternative mechanism. To begin to identify the basis for this, chylomicron remnant binding to cell membrane proteins was examined by ligand blotting. Cell extracts were prepared from each type of macrophage and the proteins were separated by SDS-PAGE and transferred to nitrocellulose paper. This was incubated with ^{125}I-labeled chylomicron remnants and binding proteins identified by autoradiography. Binding of labeled remnants to the LDL receptor of macrophages from normal and apoE-deficient mice was detected; however, the extent of binding was less for apoE-deficient macrophages (**Fig. 10**). There was no binding by macrophage extracts from LDL receptor-deficient mice. These are all consistent with the immunoblotting studies. Binding to the LRP or the VLDL receptor could not be detected, a result consistent with the conclusion that additional apoE is needed for binding to these proteins but not to the LDL receptor. However, there was a reproducible increase in remnant binding to a protein with an estimated molecular mass of 28 kDa in membranes from macrophages of apoE knockout mice when compared to those from macrophages of normal or LDL receptor knockout mice. To test the possibility that this protein might be a degradation fragment of the LRP, the LRP was immune precipitated with the polyclonal antibody and ligand blotting of the precipitate was carried out. There was no binding detectable at 28 kDa (data not shown). As this might represent a new binding protein, its cellular localization was studied. Cells were treated with pronase at low temperature to digest only surface proteins (40, 45). Solubilized extracts were prepared and the bind-

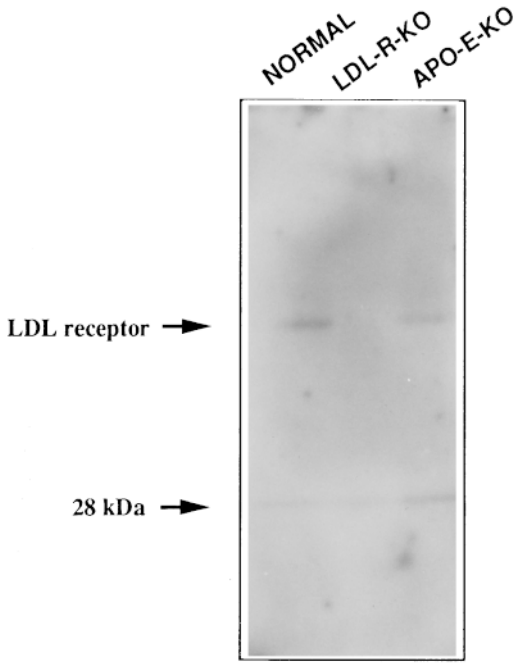

Fig. 10. Ligand blotting with radiolabeled chylomicron remnants to membrane proteins from normal, LDL receptor knockout, and apoE knockout mice. Cell extracts were prepared from macrophages from normal, LDL receptor knockout, and apoE knockout mice and equivalent amounts of protein (300 μg) were separated and transferred to nitrocellulose as described in the legend to Fig. 8. The membrane was incubated with ^{125}I-labeled chylomicron remnants (1 μg/ml) and washed, and bound radiolabeled chylomicron remnants were detected as described in Methods. NORMAL, normal mice; APO-E-KO, apoE knockout mice; LDL-R-KO, LDL receptor knockout mice. Arrows on the left correspond to the location of the LDL receptor and the 28 kDa protein. Shown is a representative autoradiogram of three separate experiments.

ing of labeled remnants by the 28 kDa protein was measured. Compared to non-pronase-treated cells, there was a reproducible decrease in remnant binding to this protein; however, the binding decreased only by 50% whereas binding to the LDL receptor was completely eliminated (not shown). These data are consistent with a presence both on the cell surface and within the cell.

Degradation and cell association of chylomicron remnants by THP-1 cells

To examine whether the LDL receptor family also plays a major role in chylomicron remnant uptake by human macrophages, the human monocyte-like cell line, THP-1, was studied. The anti-LDL receptor antibody suppressed the degradation and association of chylomicron remnants by differentiated THP-1 cells to 50% and 40%, respectively (**Fig. 11**). RAP suppressed this to 40% (not significant vs. the antibody). RAP combined with the anti-LDL receptor antibody inhibited the degradation and association to 25% (degradation, $P < 0.03$, vs. antibody). Acetyl-LDL did not inhibit the degradation or association of chylomicron remnants (93% and 101%, respectively). These results suggest that human monocyte-derived macrophages metabolize chylomicron remnants primarily by the

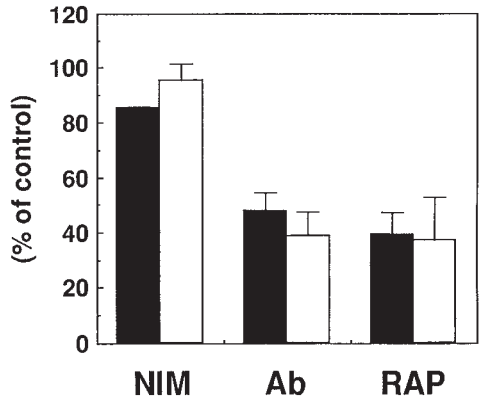

Fig. 11. Metabolism of chylomicron remnants by THP-1 macrophage-like cells. THP-1 cells were incubated with 0.1 μm PMA in medium containing 10% LPDS for 48 h to induce their differentiation into macrophages. The degradation (filled bars) and cell association (open bars) of ^{125}I-labeled chylomicron remnants in the absence and presence of 200 μg/ml non-immune IgG (NIM), 200 μg/ml anti-LDL receptor antibody (Ab), or 300 μg/ml RAP (RAP) were measured after incubation at 37°C for 4 h. The results are expressed as a percentage of the specific metabolism by cells incubated with no addition. The average specific degradation and cell association for control cells were 14.6 ± 4.2 and 47.6 ± 12.3 ng/mg cell protein (n = 4), respectively. Mean ± SE is shown.

LDL receptor and that the contribution of other members of the LDL receptor family to this process is relatively somewhat less than with primary mouse cells. This is probably due to incubation with 10% LPDS for 48 h and adaptation of the cells to culture conditions.

DISCUSSION

Several recent studies have begun to investigate the role of chylomicron remnants in the pathogenesis of atherosclerosis, and have identified a delay in their removal from serum as an independent risk factor (11–18). This raises the question of how these particles contribute to the formation of atherosclerotic lesions. Cholesterol-rich diets increase the cholesterol content of chylomicrons and their remnants. It is thus possible that they can contribute to lesion formation by serving as a lipoprotein source of cholesteryl ester possibly by facilitating the excessive accumulation of lipid in macrophages in the artery wall and thus the generation of foam cells. The mechanisms of chylomicron remnant uptake by macrophages are incompletely understood. Previously our group demonstrated that chylomicron remnants are transported by the LDL receptor pathway in J774 and P388D1 macrophage cell lines and in primary cultures of mouse peritoneal macrophages. These studies, however, also suggested that the LDL receptor does not account for all of the specific uptake (25). The studies reported here provide a quantitative estimate of the contribution of different cellular processes in chylomicron remnant metabolism and begin to test the hypothesis that chylomicron remnants can contribute directly to the transport of dietary lipid to artery wall macro-

phages and foam cell formation under conditions where atherosclerosis occurs at an accelerated rate.

To identify the potential receptor processes involved in the uptake of chylomicron remnants by macrophages, two complementary approaches were undertaken. One used specific inhibitors of the LDL receptor and the LRP and the other used macrophages harvested from normal, LDL receptor knockout, VLDL receptor knockout, and apoE knockout mice. The latter provide the unequivocal elimination of these processes and facilitate the evaluation of their roles in chylomicron remnant metabolism. There was no difference in the chylomicron remnant uptake and the inhibitory effect of the anti-LDL receptor antibody and/or RAP between resident and elicited cells. In macrophages from normal mice, anti-LDL receptor antibody inhibited remnant uptake by 40% and RAP at a relatively high concentration inhibited chylomicron remnant uptake by 60%. Together they inhibited chylomicron remnant uptake by 70%. The larger inhibition by RAP and the marginal additive effect when both RAP and the anti-LDL receptor antibody were present together suggests that the concentration of RAP used blocked not only the LRP but also the LDL receptor. Though there are some reports of the presence of the VLDL receptor on macrophages and its cell lines (56–58), the present studies suggest that any role of the VLDL receptor in remnant uptake is quantitatively minor or is compensated by LRP, because no difference in the effects of anti-LDL receptor antibody and/or RAP or in the absolute rates of degradation and association studies between normal macrophages and macrophages from VLDL receptor knockout mice were detected. Acetyl-LDL and fucoidan had no significant suppressive effect, but a reproducible decrease was observed when cells were treated with enzymes to digest cellular proteoglycans. The nature of the contribution of surface proteoglycans to remnant metabolism, particularly as it relates to cell-derived LPL and apoE, is currently being investigated. From these data, it was deduced that at least 40% of chylomicron remnant uptake is due to the LDL receptor, at least 20% due to another member of the LDL receptor family, most likely the LRP, and perhaps the remaining 20–30% due to as of yet unidentified mechanisms that do not include the scavenger receptor.

In macrophages from LDL receptor knock-out mice, the rate of chylomicron remnant uptake was reduced about half as compared to normal macrophages. This is consistent with the LDL receptor being the primary mechanism of uptake when present at normal levels. In the absence of the LDL receptor, the relative contribution of RAP-sensitive pathways increased from 20% to 50%, and the absolute rates of degradation and association of chylomicron remnants by the RAP-sensitive pathway(s) were nearly identical to those observed in normal macrophages. This was consistent with the absence of an upregulation of LRP expression in these cells. These results suggest that even in the absence of the LDL receptor, macrophages can still bind and metabolize chylomicron remnants, albeit at a slower rate. This predicts that chylomicron remnants could transport a significant amount of lipid to macrophages as LRP levels are not affected by lipid loading.

In macrophages from apoE knockout mice, chylomicron remnant uptake was not reduced compared to normal macrophages. This was not too surprising as the chylomicron remnants used were from normal animals and were apoE-enriched and thus contained an adequate concentration of this protein for binding to the LDL receptor. Consistent with the other macrophage types, the LDL receptor was a major mechanism of uptake of chylomicron remnants. The anti-LDL receptor antibody inhibited chylomicron remnant metabolism by 40%; however, this was not increased by RAP and RAP alone, at low concentration, had no effect on remnant uptake. This suggests that in the absence of apoE secretion by macrophages, the LRP and other RAP-sensitive pathways do not function. Thus it appears that apoE production is a prerequisite for the LRP to contribute to chylomicron remnant metabolism. This is analogous to β-VLDL which requires the addition of apoE to observe metabolic effects of this apoE-rich lipoprotein that are mediated via the LRP (7). A somewhat surprising observation was that LDL receptor expression was reduced in apoE-deficient macrophages. This is most likely a result of the hypercholesterolemia in apoE knockout mice and a down-regulation of macrophage LDL receptors. Alternatively, the inability of the macrophages to eliminate cellular cholesterol by an apoE-dependent mechanism could contribute to the reduction of receptor expression (59). Although LDL receptor expression was 60% of that of macrophages from normal mice, and it was concluded the LRP did not contribute to chylomicron remnant uptake by these cells, there was no difference in the absolute rate of chylomicron remnant uptake. This suggests that another mechanism of uptake is expressed and enhanced. It appears that such a pathway leads to a "normal" rate of remnant uptake despite the loss of LRP function and a decreased LDL receptor level. The mechanism could involve differences of other factors such as LPL secretion (32–34, 52, 60), the composition of membrane proteoglycans (53, 54), or the presence of another pathway such as the human triglyceride-rich lipoprotein receptor (27, 28) or another non-LDL receptor gene family member (60) and an interaction with either apoB-48 or with lipid on the lipoprotein surface. Studies of these factors as well as of the uptake of apoE-deficient chylomicron remnants are underway. In preliminary experiments it was observed that apolipoprotein-free remnant particles were taken up by cells from apoE-deficient mice and this could lead to lipid accumulation. These results are consistent with recent studies of Hendriks and colleagues (60) that VLDL from apoE-deficient mice is taken up by J774 macrophages and can stimulate cellular cholesteryl ester content.

One potential candidate mechanism that might contribute to the enhanced uptake is the binding protein that was detected by ligand blotting. The protein is located both inside the cell and on the cell surface and its level of expression is elevated in apoE deficiency. The na-

ture of the protein is not yet established but it is unlikely that it is a degradative fragment of the LRP as LRP levels were unchanged. Further, immune precipitation studies with the anti-LRP antibody were unable to precipitate any remnant binding activity from apoE knockout mouse macrophages that migrated with a similar molecular size. It is possible, because of the inverse relation between LDL receptor expression and lipoprotein binding to the 28 kDa protein, that the two may be related. However, it is more likely that the decrease in LDL receptor expression is coincidental and this is due to the pronounced hypercholesterolemia in the mice from which the macrophages were harvested. The cells were studied only 1–2 h after isolation, which is not sufficient time to overcome the inhibition of receptor expression generated in situ. In addition, the detection of binding activity at the same position on the gel in macrophages from LDL receptor knockout mice also argues against the proposal that the 28 kDa binding protein is a degradation product of the LDL receptor. More studies will be needed to evaluate the nature of the binding protein and its physiological relevance to lipoprotein metabolism.

To begin to evaluate whether chylomicron remnants can directly contribute to lipid deposition in macrophages, cells were incubated with chylomicron remnants continuously for 48 h and the extent of lipid was accumulation visualized by oil-red-O staining. Macrophages from normal mice stained intensely and most had numerous punctate-appearing globules. The staining in cells from LDL receptor and apoE knockout mice was less intense but clearly evident. Taken together with the accompanying metabolic studies, it is clear that chylomicron remnants themselves can lead to the excessive accumulation of cellular lipid. It will be of further interest to determine whether remnants promote cholesteryl ester accumulation that is induced by other lipoproteins such as oxidized LDL or LDL, especially as remnants have been shown to stimulate macrophage cholesterol synthesis (29).

In summary, the main pathway of remnant uptake in normal mouse macrophages is the LDL receptor, with LRP or another LDL receptor family member contributing to a lesser extent. The scavenger receptor is not involved. Qualitatively similar results were obtained with a human macrophage-derived cell line. In LDL receptor-deficient mice, there is a decrease in the amount of uptake and the proportion that is LRP-dependent is increased. In apoE deficiency, there is a decrease in LRP-mediated uptake, suggesting that de novo secretion of apoE from macrophage is necessary for this pathway. It is possible that the protein corresponding to the binding protein at 28 kDa is part of a compensatory mechanism, although other mechanisms must also be explored.

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