Relative importance of the LDL receptor and scavenger receptor class B in the β-VLDL-induced uptake and accumulation of cholesteryl esters by peritoneal macrophages

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Abstract **We investigated the mechanism of β-very low density lipoprotein (β-VLDL)-induced foam cell formation derived from peritoneal macrophages from control mice and low density lipoprotein (LDL) receptor-deficient mice to elucidate the role of the LDL receptor in this process. The LDL receptor appeared to be of major importance for β-VLDL metabolism. Consequently, the accumulation of cholesteryl esters in LDL receptor$^{-/-}$ macrophages is 2.5-fold lower than in LDL receptor$^{+/+}$ macrophages. In the absence of the LDL receptor, however, β-VLDL was still able to induce cholesteryl ester accumulation and subsequently we characterized the properties of this residual β-VLDL recognition site(s) of LDL receptor$^{-/-}$ macrophages. Although the LDL receptor-related protein is expressed on LDL receptor$^{-/-}$ macrophages, the cell association of β-VLDL is not influenced by the receptor-associated protein, and treatment of the macrophages with heparinase and chondroitinase was also ineffective. In contrast, both oxidized LDL (OxLDL) and anionic liposomes were able to inhibit the cell association of ^{125}I-labeled β-VLDL in LDL receptor$^{-/-}$ macrophages by 65%. These properties suggest a role for scavenger receptor class B (SR-B), and indeed, in the LDL receptor$^{-/-}$ macrophages the selective uptake of cholesteryl esters from β-VLDL was 2.2-fold higher than that of apolipoproteins, a process that could be inhibited by OxLDL, high density lipoprotein (HDL), and β-VLDL. In conclusion, the LDL receptor on peritoneal macrophages is directly involved in the metabolism of β-VLDL and the subsequent foam cell formation. When the LDL receptor is absent, SR-B appears to mediate the remaining metabolism of cholesteryl esters from β-VLDL.**—Herijgers, N., M. Van Eck, S. J. A. Korporaal, P. M. Hoogerbrugge, and T. J .C. Van Berkel. **Relative importance of the LDL receptor and scavenger receptor class B in the β-VLDL-induced uptake and accumulation of cholesteryl esters by peritoneal macrophages.** ***J. Lipid Res.*** **2000.** 41: **1163–1171.**

Supplementary key word atherosclerosis

Modified remnant lipoproteins accumulate in the plasma of cholesterol-fed animals and humans with the genetic disorder type III hyperlipoproteinemia (dysbetalipoproteinemia) (1, 2). On the basis of their electrophoretic mobilities these particles have been termed β-very low density lipoprotein (β-VLDL). β-VLDL is relatively enriched in cholesteryl esters and depleted in triglycerides. The apolipoprotein composition shows a considerable enrichment in apoE and a decreased amount of apoCs when compared with normal VLDL. Evidence of the atherogenic character of β-VLDL came from in vitro studies, in which it was shown that uptake of β-VLDL can induce cholesteryl ester accumulation in macrophages, leading to foam cell formation (3–5).

In mouse peritoneal macrophages, the uptake of β-VLDL appears to be mediated by a so-called unusual low density lipoprotein (LDL) receptor (6, 7). This unusual receptor binds LDL poorly, but binds apoE-containing lipoproteins with high affinity and is resistant to downregulation by extracellular cholesterol. Whether this receptor is identical to the classic LDL receptor as described in human fibroblasts is not fully known yet.

Studies with LDL receptor-deficient macrophages from Watanabe heritable hyperlipidemic rabbits and patients with familial hypercholesterolemia revealed that in the absence of the LDL receptor, β-VLDL can still be metabolized (8, 9). LDL receptor$^{-/-}$ macrophages, therefore, do possess a recognition site for β-VLDL.

Abbreviations: CE, cholesteryl esters; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; HDL, high density lipoprotein; HPTLC, high-performance thin-layer chromatography; LDL receptor, low density lipoprotein receptor; LRP, LDL receptor-related protein; PBS, phosphate-buffered saline; RAP, receptor-associated protein; SR-B1, scavenger receptor class B1; SR-B, scavenger receptor class B; β-VLDL, β-very low density lipoprotein.

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The most obvious candidate protein, which might function as a recognition site for β-VLDL on LDL receptor$^{-/-}$ macrophages, is the LDL receptor-related protein (LRP), possibly in concert with proteoglycans. LRP is a 600-kDa protein that shows structural homology with the ligand-binding regions of the LDL receptor (10). It recognizes apoE-containing lipoproteins in addition to various other ligands such as α_2-macroglobulin (11). The receptor-associated protein (RAP) blocks the binding of all known ligands to LRP (12). Wu and Pizzo (13) showed that LRP is expressed on murine peritoneal macrophages. Proteoglycans are proteins that have one or more attached glycosaminoglycan chains, with highly negatively charged sulfate and carboxylate groups. A large number of ligands are known to bind to proteoglycans, including apoE-enriched lipoproteins (14). It has been suggested that proteoglycans and LRP might act in concert to allow lipoprotein uptake (15). The role of other candidate receptors in the uptake of β-VLDL, such as the VLDL receptor (16, 17) and the lipolysis-stimulated receptor (18), is unclear at the moment.

In the present study, we used LDL receptor-containing and LDL receptor-negative macrophages to determine the exact role of the LDL receptor in the catabolism of β-VLDL. Furthermore, the LDL receptor-negative macrophages were utilized to characterize the recognition site(s) that enable(s) the residual β-VLDL catabolism by these cells.

METHODS

Mice

Homozygous LDL receptor knockout (LDL receptor$^{-/-}$) mice, originally generated by Ishibashi et al. (19), were obtained from the Jackson Laboratory (Bar Harbor, ME) as mating pairs, and bred in the Gaubius Laboratory (Leiden, The Netherlands). These mice were hybrids between the C57BL/6 and 129 Sv strains (F_4 generation of backcrosses to C57BL/6). C57BL/6 mice were obtained from Broekman (Someren, The Netherlands). They were fed standard rat/mouse chow (SMR-A; Hope Farms, Woerden, The Netherlands). The experiments were approved by the ethics committee on animal experiments of Leiden University (Leiden, The Netherlands).

Isolation and labeling of lipoproteins

β-VLDL was obtained from rats that were fed a diet containing 2% cholesterol, 5% olive oil, and 0.5% cholic acid for 2 weeks. The rats were fasted overnight, after which blood was collected by puncture of the abdominal aorta. LDL and high-density lipoprotein (HDL) were obtained from the sera of healthy human volunteers. The sera were centrifuged at 250,000 *g* in a discontinuous KBr gradient for 18 h as reported by Redgrave, Roberts, and Wert (20). The lipoproteins were dialyzed against phosphate-buffered saline (PBS) containing 1 mM EDTA. The composition of β-VLDL was 14.6 ± 2.1% triacylglycerols, 15.8 ± 1.1% phospholipids, 49.4 ± 3.1% cholesteryl esters, 9.9 ± 1.0% free cholesterol, and 10.3 ± 0.7% protein.

The apolipoprotein composition was as follows: apoB-100, 25 ± 2%; apoB-48, 10 ± 2%; apoA-IV, 1 ± 0%; apoE, 42 ± 4%; apoA-I; 10 ± 2%; apoA-II, 2 ± 1% and apoCs, 10 ± 3%.

LDL was acetylated with repeated additions of acetic anhydride according to Basu et al. (21). HDL was passed through a heparin-Sepharose affinity column to remove apoE-containing particles. Cu^{2+} oxidation of LDL was performed as described previously (22).

β-VLDL was labeled with ^{125}I or ^{125}I-labeled tyramine cellobiose at pH 10.0 according to McFarlane (23), as modified by Nagelkerke, Barto, and van Berkel (24). Free ^{125}I was removed by Sephadex G50 gel filtration, followed by dialysis against PBS containing 1 mM EDTA.

The label distribution among the various apolipoproteins was as follows: apoB, 34 ± 4%; apoA-IV, 2 ± 0%; apoE, 9 ± 3%; apoA-I, 12 ± 4%; and apoCs, 43 ± 3%.

β-VLDL was labeled with 1α,2α-(*n*)-[^{3}H]cholesteryl linoleate ([^{3}H]CE) or 1α,2α-(*n*)-[^{3}H]cholesteryl oleoyl ether (Amersham, Amersham, UK) by exchange of donor particles as reported previously (25). In brief, donor particles were formed by sonication of egg yolk phosphatidylcholine (Fluka, Buchs, Switzerland) supplemented with 50 μCi of [^{3}H]CE. Sonication was carried out with an MSE Soniprep 150 (amplitude, 12 μm; MSE Scientific Instruments, Crawley, UK) for 15 min at 52°C under a constant stream of argon in a 0.1 M KCl–10 mM Tris–1 mM EDTA–0.025% NaN_3 buffer, pH 8.0. Donor particles with a density of 1.03 g/mL were isolated by density gradient ultracentrifugation. Phospholipid content of the particles was measured by an enzymatic colorimetric assay (Boehringer GmbH, Mannheim, Germany). β-VLDL was labeled by incubating β-VLDL with donor particles (β-VLDL-protein: particle phospholipid mass ratio, 8:1) in the presence of human lipoprotein-deficient serum as cholesteryl ester transfer protein source 1:1 (v/v) for 8 h at 37°C in a shaking water bath under argon. Ethylmercurithiosalicylate (thimerosal, 20 mM; Sigma, St. Louis, MO) was added to stimulate cholesteryl ester transfer and to inhibit phospholipid transfer and lecithin–cholesterol acyltransferase activity (26). Radiolabeled β-VLDL was then isolated by density gradient ultracentrifugation and dialyzed against PBS–1 mM EDTA. The effect of the labeling procedure on β-VLDL was analyzed by measurement of phospholipid, cholesterol, cholesteryl ester, and triglyceride content (enzymatic kits; Boehringer GmbH). Labeled β-VLDL was used only when no differences compared with the original unlabeled β-VLDL batch were detected.

The [^{3}H]cholesteryl oleoyl ether and ^{125}I-labeled tyramine cellobiose double-labeled β-VLDL was fractionated into two size populations by consecutive density gradient ultracentrifugation steps in a discontinuous KBr gradient as reported by Redgrave et al. (20). After centrifugation for 2 h at 40,000 rpm, the top layer (fraction 1) was removed by aspiration and replaced by KBr buffer of d 1.006 g/ml. After another 4 h of centrifugation at 40,000 rpm, a second fraction was obtained as top layer (fraction 2).

The sizes of fractions 1 and 2 were analyzed by dynamic light scattering (4700 system; Malvern Instruments, Southboro, MA) and were, respectively, 93 and 49 nm. The ^{3}H/^{125}I ratio was, for fraction 1, 1.03 and for fraction 2 a value of 0.92 was obtained, as compared with the defined value of 1.00 for the total β-VLDL fraction.

α_2-Macroglobulin, GST–RAP, and lactoferrin

A plasmid (pGEX) encoding a fusion protein (GST–RAP) of glutathione *S*-transferase (GST) and the 39-kDa protein of receptor-associated protein (RAP), which was transformed in *Escherichia coli* (DH5α), was a generous gift of J. Herz (Dallas, TX). GST–RAP was produced as described by Herz et al. (12). Transformed *E. coli* were cultured at 37°C to an OD_{600} of 0.4–0.5, transcription of GST–RAP was induced with isopropyl-β-D-thiogalactopyranoside (0.01%), and growth was continued for 6 h at 37°C. Cells were harvested by centrifugation at 4°C and GST–RAP was isolated from the solubilized cells with GSH-Sepharose. The protein content of the isolated GST–RAP was determined by the method of Lowry et al. (27) with bovine serum albumin (BSA) as standard. The GST–RAP solution was subsequently

concentrated to a concentration of 10 mg of GST–RAP per mL, using polyethylene glycol 8000. The isolated GST–RAP was more than 95% pure as determined by analysis by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Human α_2-macroglobulin (α_2-M) was isolated as described previously (28) and radioiodinated with chloramine-T as published previously (29). In brief, α_2-M (350 μg/mL) in 200 μl of NaCl–P_i, pH 8.0, was mixed with 8 μl of ^{125}I in 0.1 M NaOH followed by 214 μg of chloramine-T in 107 μl of NaCl–P_i, pH 8.0. After 90 s, the reaction was stopped by the addition of 164 μg of $Na_2S_2O_5$ in 82 μl of NaCl–P_i, pH 8.0, and 120 μg of KI in 60 μl of NaCl–Pi, pH 8.0. In a subsequent step, ^{125}I-labeled α_2-M was directly activated with trypsin by incubation with a 15-fold molar excess of trypsin for 5 min at 20°C followed by a 5-fold molar excess of soybean trypsin inhibitor as related to trypsin (29). Activated ^{125}I-labeled α_2-M was separated from smaller protein complexes by gel filtration on a 0.7 × 25 cm Bio-Gel A-1.5m column with PBS–1 mM EDTA, pH 7.4.

Human lactoferrin (iron satured) was obtained from Serva (Heidelberg, Germany).

Phospholipid liposome preparation

Unilamellar liposomes were obtained by sonication of egg yolk phosphatidylcholine (Fluka):phosphatidylserine (Sigma): cholesterol (Sigma) in a molar ratio of 1:1:1. The lipids were mixed in chloroform and dried under a stream of N_2. Sonication was carried out with an MSE Soniprep 150 for 40 min (amplitude, 12 μm) at 52°C under a constant stream of argon in a 0.1 M KCl–10 mM Tris–1 mM EDTA–0.025% NaN_3 buffer, pH 8.0. Particles with a density of 1.03 g/mL were isolated by density gradient ultracentrifugation and dialyzed against PBS–1 mM EDTA. The liposomes were stored at 4°C under argon. The average diameter of the liposomes was determined by photon correlation spectroscopy (System 4700 C; Malvern Instruments).

Peritoneal macrophage harvesting

Five days after peritoneal injection of 1 mL of 3% Brewer thioglycollate medium (Difco, Detroit, MI), peritoneal macrophages were harvested by lavage of the peritoneal cavity with 10 mL of PBS–1 mM EDTA. After three washing steps, the cells (0.5×10^6) were plated out in 25-mm multiwell culture dishes with Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Verviers, Belgium) containing 10% fetal calf serum (FCS), 2 mM glutamine, streptomycin (100 μg/mL), and penicillin (100 IU/mL). After 4 h the nonadherent cells were removed by washing and the culture medium was replaced by DMEM containing 10% human lipoprotein-deficient serum, 2 mM glutamine, streptomycin (100 μg/mL), and penicillin (100 IU/mL). The cells were cultured for 2 days.

Metabolic studies with cultured murine peritoneal macrophages

Association and degradation studies were carried out with the indicated amounts of ^{125}I-labeled protein for 3 h at 37°C. When indicated, the cells were treated with heparinase (2.4 U/mL; Sigma) or chondroitinase (0.24 U/mL; Sigma) for 40 min at 37°C before the experiment, after which the cells were washed. Incubations of the cells were performed in DMEM (BioWhittaker), containing 2% (w/v) BSA (Sigma) in a total volume of 0.5 mL. After incubation, the cells were washed three times with washing buffer (Tris-HCl [50 mmol/L, pH 7.4], containing 0.9% NaCl, EDTA [1 mmol/L], $CaCl_2$ [5 mmol/L], and 0.2% [w/v] BSA) followed by two washing steps with washing buffer without BSA. The cells were lysed in NaOH (0.1 mol/L) and the radioactivity was determined. Cell protein was measured by the method of Lowry et al. (27) with BSA as standard. Degradation of the radiolabeled protein was determined as follows: to 0.5 mL of the medium, 0.4 mL of 35% trichloroacetic acid and 10 μL of 20% KI were added (30). The mixture was incubated for 30 min at 4°C and subsequently 0.25 mL of 0.7 M $AgNO_3$ was added. After centrifugation (5 min at 16,000 *g*), the radioactivity in the supernatant was determined. The maximal association and degradation were calculated with Graphpad (San Diego, CA) Prism software.

Cellular accumulation of lipid

Murine peritoneal macrophages were cultured as described above. At the start of the experiment fresh DMEM containing 2% BSA in the presence or absence of lipoprotein (50 or 100 μg/mL) was added and the cells were incubated at 37°C for 24 h. The cells were then washed three times with PBS containing 0.1% BSA, followed by two washing steps with PBS alone. Intracellular lipid was determined as described by Havekes, de Wit, and Princen (31). Briefly, the cells were harvested by scraping with a rubber policeman and resuspended by three successive slow passages through a syringe needle (25 gauge). Samples (100 μL) were taken for measurement of protein. Lipids were extracted from the cell suspension with methanol–chloroform 2:1 (v/v) as described by Bligh and Dyer (32). Cholesteryl acetate (2 μg) was added as an internal standard. The lipids were separated by high-performance thin-layer chromatography (HPTLC). The lipid bands were quantified densitometrically on a Shimadzu (Kyoto, Japan) CS910 chromatograph scanner at 380 nm and areas under the curve were integrated with a data processor.

Statistical analysis

Statistical analysis of the data was performed with the unpaired Student *t*-test.

RESULTS

Role of macrophage LDL receptor in β-VLDL catabolism and foam cell formation

To determine the exact role of the macrophage LDL receptor in β-VLDL catabolism, we examined the cell association and degradation of β-VLDL by the peritoneal macrophages from LDL receptor-negative and control mice. As shown in **Fig. 1**, LDL receptor-containing macrophages appeared to be much more efficient in the association and degradation of β-VLDL than LDL receptor-negative macrophages (B_{max} = 3,730 vs. 797 ng/mg for association [Fig. 1A] and B_{max} = 2,163 vs. 202 ng/mg for degradation [Fig. 1B]). These results indicate that the LDL receptor on peritoneal macrophages plays a prominent role in the metabolism of β-VLDL.

To determine whether the metabolism of β-VLDL via the LDL receptor results in cholesteryl ester (CE) accumulation, we compared the CE accumulation capacity of peritoneal macrophages isolated from LDL receptor-negative and control mice. In the absence of added lipoprotein, no statistically significant difference in the amount of CE between the LDL receptor-positive and -negative macrophages could be observed (2.33 ± 0.76 vs. 3.97 ± 1.05 μg of cholesteryl ester per mg of cell protein; $P < 0.35$) (**Fig. 2**). Incubation of the cells with β-VLDL (50 μg/mL) for 24 h, however, induced in the LDL receptor-containing macrophages an 8.8-fold ($P < 0.002$) increase in CE accumulation; this factor was 1.9 ($P < 0.025$) for the LDL receptor-negative macrophages. Thus, the CE accumulation in the LDL

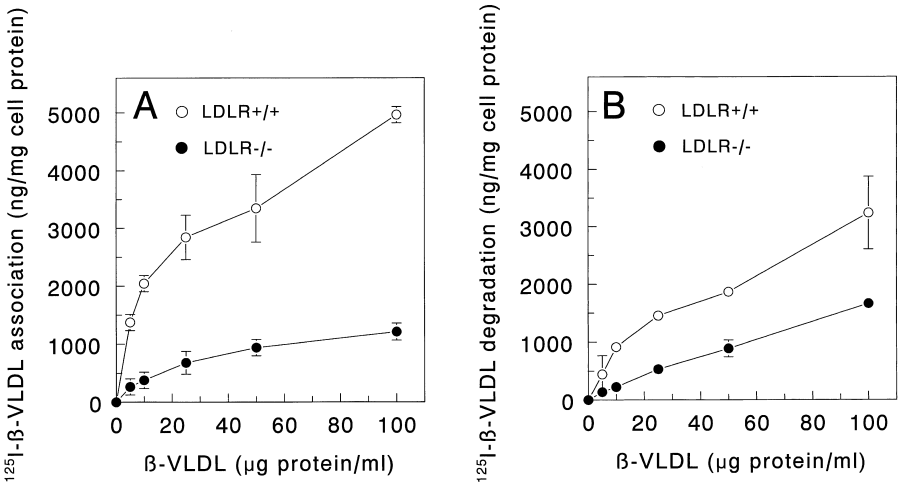

Fig. 1. Effect of increasing ^{125}I-labeled β-VLDL concentrations on cell association and degradation by LDL receptor$^{+/+}$ and LDL receptor$^{-/-}$ peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages of LDL receptor$^{+/+}$ mice (open circles) and LDL receptor$^{-/-}$ mice (solid circles) were isolated and cultured in lipoprotein-deficient medium for 2 days. The association (A) and degradation (B) of ^{125}I-labeled β-VLDL by the macrophages (3 h at 37°C) were determined and expressed as nanograms of ^{125}I-labeled β-VLDL per mg of cell protein. The data are the results of three independent experiments. Values represent means ± SD.

receptor-containing macrophages was higher than in LDL receptor-negative macrophages. Incubation of the cells with acetylated LDL (AcLDL, 50 µg/mL) did not result in a different amount of CE in the two cell types, indicating that the scavenger receptor class A-mediated accumulation of CE is not different for the LDL receptor-containing macrophages as compared with LDL receptor-negative macrophages. With AcLDL (100 µg/mL), values for, respectively, LDL receptor-negative and -positive macrophages were 10.2 ± 0.3 and 11.6 ± 2.1 µg of cholesteryl ester per mg of cell protein, indicating that for AcLDL as compared with β-VLDL, relatively higher concentrations are needed for significant cholesteryl ester accumulation.

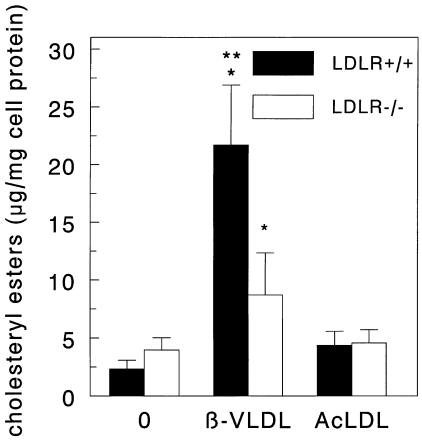

Fig. 2. Effect of β-VLDL and AcLDL on cholesteryl ester accumulation in LDL receptor$^{+/+}$ and LDL receptor$^{-/-}$ peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages of LDL receptor$^{+/+}$ mice (solid bars) and LDL receptor$^{-/-}$ mice (open bars) were isolated and cultured in lipoprotein-deficient medium. After incubation with β-VLDL or AcLDL (50 µg/mL) for 24 h at 37°C, the cellular cholesteryl ester content was measured and expressed as micrograms of cholesterol ester per mg of cell protein. The data represent the results of three independent experiments. Values represent means ± SD. * Significant difference versus control macrophages in the absence of β-VLDL ($P \leq 0.002$ for LDL receptor$^{+/+}$ macrophages and $P \leq 0.025$ for LDL receptor$^{-/-}$ macrophages); ** significant difference versus LDL receptor$^{-/-}$ macrophages in the presence of β-VLDL ($P \leq 0.023$).

Characterization of β-VLDL recognition site(s) on LDL receptor$^{-/-}$ peritoneal macrophages

Although the LDL receptor appears to be of major importance for the accumulation of CE from β-VLDL, the residual accumulation in the absence of the LDL receptor still appears significant. This stimulated us to characterize recognition site(s) that is (are) responsible for β-VLDL metabolism of peritoneal macrophages in the absence of the LDL receptor.

The first candidate receptor we tested was the LDL receptor-related protein (LRP). The association of a well-known ligand for LRP, ^{125}I-labeled α_2-macroglobulin (11) (2.6 µg/mL), with LDL receptor$^{-/-}$ peritoneal macrophages was inhibited by more than 70% by GST–RAP (5 µg/mL), which is a molecular chaperone and inhibitor that binds tightly to LRP (12) (389 ± 37 vs. 116 ± 11 ng of ^{125}I-labeled α_2-M per mg of cell protein). LRP, thus, is expressed on LDL receptor$^{-/-}$ peritoneal macrophages. GST–RAP, however, was not able to inhibit the association and degradation of ^{125}I-labeled β-VLDL (**Fig. 3**).

We then tested the possible involvement of proteoglycans in the association of ^{125}I-labeled β-VLDL to LDL receptor$^{-/-}$ macrophages. Both heparinase treatment (2.4 U/mL) as well as chondroitinase treatment (0.24 U/mL) did not influence the association of ^{125}I-labeled β-VLDL to LDL receptor$^{-/-}$ macrophages (data not shown). Preincubation of the cells with 10-fold higher concentrations of the enzymes gave similar results (data not shown). Furthermore, the association of ^{125}I-labeled β-VLDL could not be blocked by more than 20% with lactoferrin, an Fe^{3+}-carrying protein with an Arg + Lys-rich sequence at positions 25–31, which binds to proteoglycans (33) (data not shown).

Subsequently we performed competition studies with ligands for scavenger receptors class A (AcLDL) and oxidized LDL (OxLDL) and scavenger receptors class B (OxLDL and anionic liposomes). The association and degradation of ^{125}I-labeled β-VLDL by LDL receptor$^{-/-}$ macrophages were effectively inhibited by β-VLDL itself (75% inhibition of association [**Fig. 4A**] and more than 80% for the degradation [Fig. 4B]), indicating it was indeed a high-

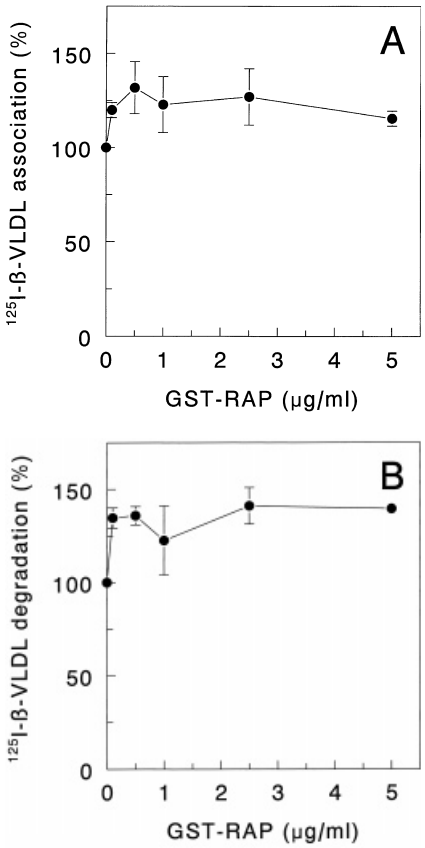

Fig. 3. Effect of GST–RAP on cell association and degradation of ^{125}I-labeled β-VLDL to LDL receptor$^{-/-}$ peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages of LDL receptor$^{-/-}$ mice were isolated and cultured in lipoprotein-deficient medium for 2 days. The cells were incubated for 3 h at 37°C with ^{125}I-labeled β-VLDL (5 μg/mL) and the indicated concentrations of GST–RAP. The 100% value for association (A) and degradation (B) of ^{125}I-labeled β-VLDL in the absence of GST–RAP was, respectively, 248.4 ± 4.3 and 136.9 ± 14.4 ng/mg of cell protein. The data represent the results of three independent experiments. Values represent means ± SD.

affinity site. LDL and AcLDL showed only a slight inhibition at a relatively high excess concentration (29 and 33% inhibition of cell association, respectively; Fig. 4A). In contrast to LDL and AcLDL, OxLDL was a highly effective inhibitor; even at 5 and 10 μg of OxLDL per mL, the cell association (Fig. 4C) and degradation (Fig. 4D) of ^{125}I-labeled β-VLDL were inhibited by 60–65% and 50–55%, respectively. Coincubation of OxLDL with poly(I) hardly influenced the inhibitory action of OxLDL. Poly(I) does block the interaction of OxLDL with scavenger receptor class A (22) and the persistent inhibition of OxLDL in the presence of poly(I) thus indicates that the effect of OxLDL was not due to interaction with a poly(I)-sensitive site as is found with scavenger receptor class A. The observed inhibition profile is consistent with a potential role of scavenger receptor class B (SR-B) (34, 35).

Rigotti, Acton, and Krieger (36) showed that anionic phospholipid liposomes effectively interact with scavenger receptor class B whereas neutral liposomes are ineffective. We thus studied the effect of these liposomes. Liposomes containing phosphatidylcholine, cholesterol, and the anionic phospholipid phosphatidylserine inhibited association of ^{125}I-labeled β-VLDL by 64% (**Fig. 5A**), whereas neutral liposomes, consisting of only phosphatidylcholine and cholesterol, were much less efficient. Also, the degradation of β-VLDL (Fig. 5B) was specifically inhibited by the phosphatidylserine liposomes.

Because these results suggest the involvement of SR-B we tested whether LDL receptor$^{-/-}$ peritoneal macrophages were able to selectively take up cholesteryl esters from β-VLDL. On LDL receptor$^{-/-}$ cells, the apparent association of [^{3}H]CE–β-VLDL, as calculated according to Pittman et al. (37), could be significantly inhibited by OxLDL, β-VLDL, and HDL (54, 51, and 46% inhibition, respectively [$P < 0.01$]), but not by LDL and AcLDL (**Fig. 6**). On LDL receptor$^{+/+}$ macrophages, β-VLDL was the only inhibitor of association of [^{3}H]CE–β-VLDL. In addition, the ratio of [^{3}H]CE–β-VLDL to ^{125}I-labeled β-VLDL, and thus of CE uptake versus particle uptake, was higher in LDL receptor$^{-/-}$ macrophages than in LDL receptor$^{+/+}$ macrophages (2.1 vs. 1.5).

The selective uptake ratio was also determined by using double-labeled [^{3}H]cholesteryl oleyol ether and ^{125}I-labeled tyramine cellobiose β-VLDL. The ratios of cholesteryl ether to apolipoprotein for LDL receptor-negative and -positive cells were, respectively, 2.3 ± 0.1 and 1.6 ± 0.1 (±SE; n = 3), confirming the presence of a selective cholesteryl ether uptake route from β-VLDL in the peritoneal macrophages.

In theory, the cellular association of a subclass of β-VLDL with a higher ratio of cholesterol ester to protein might lead to an apparent selective uptake value higher than 1. We have separated by sequential centrifugation the β-VLDL into two fractions (see Materials and Methods) with mean sizes of 93 and 49 nm and determined ^{3}H/^{125}I ratios for these fractions of, respectively, 1.03 and 0.92, which indicates that a variation in the ratio of cholesteryl ester to apoprotein between subclasses of β-VLDL cannot explain our observed selective uptake values.

DISCUSSION

Macrophage-derived foam cells play an important role in the initiation and progression of atherosclerosis (38). One of the best characterized mechanisms by which macrophages in the vessel wall become foam cells is by uptake of modified LDL via the scavenger receptor-mediated pathway (39). The exact role of the LDL receptor in the formation of atherosclerosis is not fully clear yet, as it is assumed that downregulation of the LDL receptor by cellular cholesterol will limit its role in foam cell formation (40). The expression of the LDL receptor on macrophages, however, may be more resistant to downregulation by cholesterol than is LDL receptor expression on fibroblasts, as suggested by studies by Koo, Wernett-Hammond, and Innerarity (6) and Ellsworth, Kraemer, and Cooper (7). Furthermore, the expression of the LDL receptor in vitro was demonstrated on foam cells extracted from the human artery wall (41).

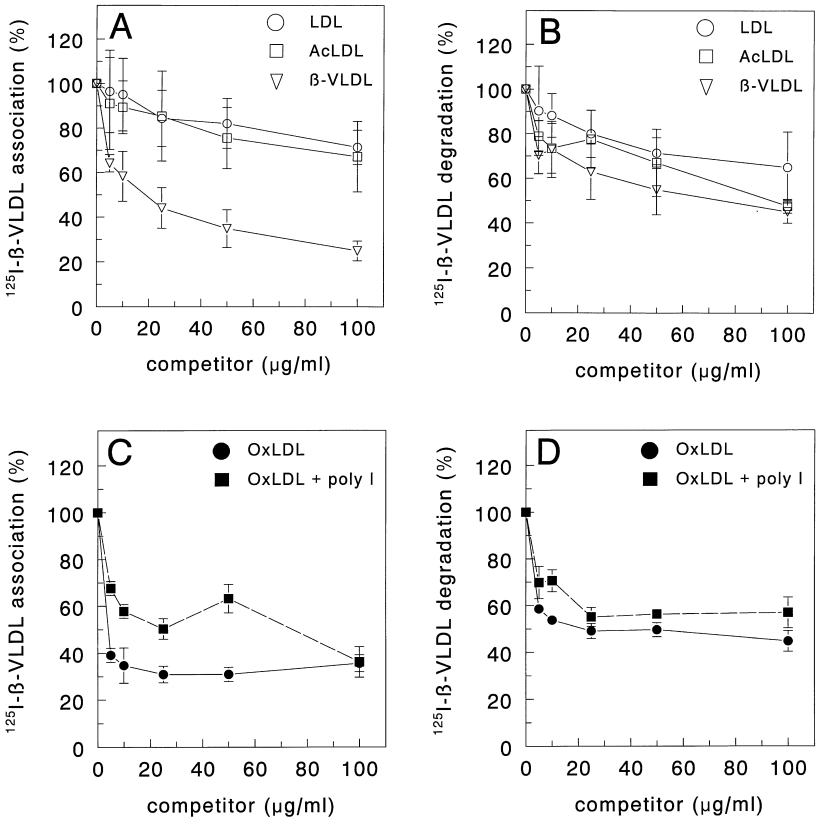

Fig. 4. Effect of increasing concentrations of LDL, AcLDL, OxLDL [± poly(I)], and β-VLDL on the cell association and degradation of ^{125}I-β-VLDL to LDL receptor$^{-/-}$ peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages of LDL receptor$^{-/-}$ mice were isolated and cultured in lipoprotein-deficient medium for 2 days. The cells were incubated for 3 h at 37°C with ^{125}I-labeled β-VLDL (5 μg/mL) with the indicated concentrations of competitor. (A) and (B) show the competitors LDL (open circles), AcLDL (open squares), or β-VLDL (inverted open triangles). (C) and (D) show OxLDL (solid circles) or OxLDL plus poly(I) (100 μg/mL) (solid squares). The 100% value for association (A and C) and degradation (B and D) of ^{125}I-labeled β-VLDL were, respectively, 248.4 ± 4.3 and 162.6 ± 25.9 ng/mg of cell protein. The data represent the results of three independent experiments. Values represent means ± SD.

In the present study, we investigated the role of the LDL receptor in the process of foam cell formation by using LDL receptor$^{-/-}$ and LDL receptor$^{+/+}$ murine peritoneal macrophages. Incubation of the LDL receptor$^{+/+}$ cells with atherogenic β-VLDL resulted in a 2.5-fold higher accumulation of cholesteryl esters when compared with LDL receptor$^{-/-}$ cells, indicating that the LDL receptor is involved in β-VLDL metabolism and the subsequent accumulation of cholesteryl esters. The finding that incubation with AcLDL did not reveal any significant difference between LDL receptor$^{+/+}$ and LDL receptor$^{-/-}$ macrophages excludes the possibility that a varying expression of scavenger receptor class A in the two cell types might explain the observed difference. In addition, these results prove that the so-called unusual LDL receptor (6, 7) originates from the same gene encoding the classic LDL receptor described in human fibroblasts.

Bone marrow transplantation studies in our laboratory support these findings. The transplantation of LDL receptor$^{-/-}$ bone marrow into irradiated LDL receptor$^{+/+}$ mice resulted in a significant reduction of the mean atherosclerotic lesion area without affecting serum cholesterol levels, suggesting that the effects of the LDL receptor on macrophages in atherosclerosis are mainly direct (42). Similar results were reported by Linton et al. (43)

The present results of our experiments demonstrate that it is possible to induce cholesteryl ester accumulation by β-VLDL in LDL receptor$^{-/-}$ macrophages, although

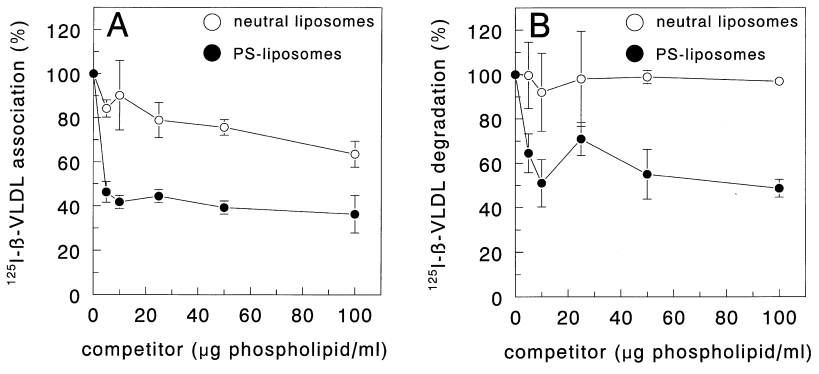

Fig. 5. Effect of increasing concentrations of phosphatidylserine liposomes and neutral liposomes on the cell association and degradation of ^{125}I-labeled β-VLDL to LDL receptor$^{-/-}$ peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages of LDL receptor$^{-/-}$ mice were isolated and cultured in lipoprotein-deficient medium for 2 days. The cells were incubated for 3 h at 37°C with ^{125}I-labeled β-VLDL (5 μg/mL) with the indicated concentrations of phosphatidylserine liposomes (solid circles) or neutral liposomes (open circles). The 100% value for association (A) and degradation (B) of ^{125}I-labeled β-VLDL in the absence of liposomes was, respectively, 243.4 ± 23.9 and 179.4 ± 4.1 ng/mg of cell protein. The data represent the results of three independent experiments. Values represent means ± SD.

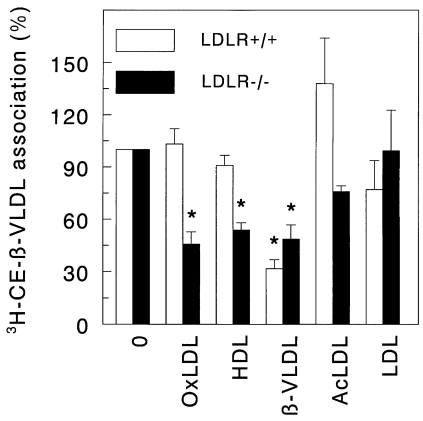

Fig. 6. Effect of native and modified lipoproteins on the cell association of [^{3}H]CE–β-VLDL to LDL receptor$^{-/-}$ and LDL receptor$^{+/+}$ peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages of LDL receptor$^{-/-}$ mice (solid columns) and LDL receptor$^{+/+}$ mice (open columns) were isolated and cultured in lipoprotein-deficient medium for 2 days. The cells were incubated for 3 h at 37°C with [^{3}H]CE–β-VLDL (1 μg/mL) with either OxLDL (50 μg/mL), HDL (100 μg/mL), β-VLDL (100 μg/mL), AcLDL (100 μg/mL), or LDL (100 μg/mL). The 100% value for the apparent association of [^{3}H]CE–β-VLDL in the absence of competitors was 970.4 ± 147.8 ng/mg of cell protein for LDL receptor$^{+/+}$ cells and 254.98 ± 38.6 ng/mg of cell protein for LDL receptor$^{-/-}$ cells. The data represent the results of three independent experiments. * Significant difference versus no competitors, $P < 0.01$.

less efficiently than in LDL receptor$^{+/+}$ macrophages. Similar results with LDL receptor-deficient macrophages from Watanabe heritable hyperlipidemic rabbits and familial hypercholesterolemic patients were obtained by Van Lenten et al. (8, 9).

As a next step, we characterized the site responsible for β-VLDL metabolism on LDL receptor$^{-/-}$ macrophages. The first candidate protein we tested was LRP. The importance of LRP was demonstrated by experiments in which inactivation of LRP in LDL receptor$^{-/-}$ mice by either conditional gene targeting or adenoviral overexpression of the blocking ligand RAP resulted in a massive accumulation of remnant lipoproteins (44, 45). Although LRP was expressed on LDL receptor$^{-/-}$ macrophages we could not observe an inhibitory role of RAP, leading to the conclusion that LRP is not responsible for β-VLDL metabolism in LDL receptor-negative macrophages. The absence of an effect of heparinase or chondroitinase treatment of the cells on β-VLDL catabolism indicated that proteoglycans are also not involved.

The results of the competition experiments strongly suggest the involvement of SR-B in the metabolism of β-VLDL by LDL receptor$^{-/-}$ macrophages. The class B scavenger receptors comprise scavenger receptor BI and II, CD36, and croquemort (46). A unique feature of members of scavenger receptor class B is their broad substrate specificity, which includes HDL, LDL, VLDL, anionic phospholipids, and apoptotic cells (36, 46, 47). In the present study, both OxLDL and anionic liposomes were already at relatively low concentrations effective inhibitors of the association of ^{125}I-labeled β-VLDL. The observation that poly(I) hardly influenced the inhibitory action of OxLDL excludes an important role of scavenger receptor class A. Similarly, Sakaguchi et al. (48) found only a slight reduction in the formation of atherosclerotic lesions in scavenger receptor class A/LDL receptor double-knockout mice when compared with LDL receptor$^{-/-}$ mice. Another characteristic feature of SR-B is the selective uptake of cholesteryl esters (49). We demonstrate that the LDL receptor$^{-/-}$ macrophages indeed show selective uptake of cholesteryl esters from β-VLDL, and that this could be inhibited by OxLDL, HDL, and β-VLDL. By comparing particle uptake versus cholesteryl ester uptake, we conclude that SR-B, thus, is relatively more important on LDL receptor$^{-/-}$ macrophages than on LDL receptor-positive macrophages. Among the various types of scavenger receptor class B, both CD36 and scavenger receptors BI and BII have been shown to facilitate selective cholesteryl ester uptake from HDL (50–52). The 2.1–2.3 ratio of cholesteryl ester to apolipoprotein for the interaction of β-VLDL with LDL receptor-negative macrophages is relatively low compared with values obtained under similar conditions for the interaction of HDL with rat liver parenchymal cells (5.6 with a similar 3 h of incubation [see ref. 35]). However, for β-VLDL no comparable values are available and as the binding specificity or lack of specificity of CD36 and scavenger receptor BI and BII are comparable, the present data do not allow a further specification among the different forms of scavenger receptor class B.

Our results, thus, strongly indicate the involvement of SR-B on macrophages in the metabolism of β-VLDL. SR-BI is mainly expressed in liver and steroidogenic tissues (49); its expression has also been shown on several types of macrophages and foam cells (53, 54). SR-BI plays a role in the final stage of reverse cholesterol transport by delivering HDL-cholesterol to the liver (49, 55, 56). Evidence indicates that SR-BI can also be involved in the removal of cholesterol from macrophages or foam cells in atherosclerotic plaques (53). CD36 is expressed on monocyte/macrophages, platelets, adipocytes, mammary epithelial cells, microvascular endothelial cells, and hepatic sinusoidal cells (57, 58). Its role in lipoprotein metabolism was suggested on the basis of its interaction with oxidized LDL (59) whereas the interaction with the native lipoproteins HDL, LDL, and VLDL was more recently established (60). The function of scavenger receptor class B in mediating the uptake of cholesteryl ester from remnant lipoproteins by macrophages points thus to a new function in the development of atherosclerosis.

The net effect of a change in SR-B expression on macrophages for cholesteryl ester uptake versus cholesterol release is unclear yet and may depend on the local remnant versus HDL concentration. Further studies with macrophage-specific SR-BI and CD36 knockout animals are needed to specify or quantify the relative role of SR-BI and CD36 in cholesteryl ester uptake from β-VLDL and its pro- and antiatherogenic role in macrophages. Bone marrow transplantation experiments, similar to those performed earlier for analysis of the role of macrophage-specific LDL receptor and apoE (61, 62), could also be of further help

in the elucidation of the role of SR-BI and CD36 on macrophages in atherosclerotic lesion formation.

In conclusion, we have shown that the LDL receptor on peritoneal macrophages is directly involved in the metabolism of β-VLDL and is of major importance for cholesteryl ester accumulation. In the absence of the LDL receptor, the competition studies and selective cholesteryl ester uptake data point to SR-B as the residual site responsible for β-VLDL metabolism. Further studies are required to reveal the exact role of SR-B expression on macrophages in the atherosclerotic process and its dualistic role in selective cholesteryl ester uptake and cholesterol release.

This research was supported by The Netherlands Heart Foundation (project 93-188). The authors thank Carla Vogelezang for expert technical assistance and Dr. Bert-Jan Ziere for helpful discussion. They are also grateful to J. Herz for providing the plasmid (pGEX) encoding GST–RAP.

Manuscript received 16 December 1998 and in revised form 2 March 2000.

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