Relative roles of LDLr and LRP in the metabolism of chylomicron remnants in genetically manipulated mice

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Abstract **Remnant-like emulsions labeled with cholesteryl [^{13}C]-oleate were prepared with lipid compositions similar to remnants derived from triacylglycerol-rich lipoproteins. When injected into the bloodstream of conscious mice, the remnant-like emulsions were metabolized in the liver leading to the appearance of $^{13}CO_2$ in the breath. Previously, using this technique, we found that remnant metabolism was significantly impaired but not completely inhibited in mice lacking low density lipoprotein receptors (LDLr). We have now found in mice with non-functional low density lipoprotein receptor-related protein (LRP) that breath enrichment of $^{13}CO_2$ was significantly decreased, indicating that the LRP also plays an important role in the metabolism of chylomicron remnants (CR). The enrichment of $^{13}CO_2$ in the expired breath was negligible in mice lacking both LDLr and receptor-associated protein (−/−), essential for normal function of LRP. In mice pre-injected with gluthatione *S*-transferase–receptor-associated protein to block LRP binding, there was a marked inhibition of the appearance of $^{13}CO_2$ in the expired breath of homozygous LDLr-deficient mice, supporting the role of LRP in vivo. Whether or not LDLr were present, in mouse and human fibroblast cells human apoE3 or E4 but not apoE2 were essential for binding of remnant-like emulsions, while lactoferrin and suramin completely inhibited binding. We conclude that in normal mice LDLr are important for the physiological metabolism of CR. When LDLr are absent the evidence supports a role for the LRP in the uptake of CR in liver cells and in fibroblasts, with binding characteristics for CR-associated apoE similar to LDLr.**—Martins, I. J., E. Hone, C. Chi, U. Seydel. R. N. Martins, and T. G. Redgrave. **Relative roles of LDLr and LRP in the metabolism of chylomicron remnants in genetically manipulated mice.** ***J. Lipid Res.*** **2000.** 41: **205–213.**

Supplementary key words chylomicron remnant • emulsion • cholesteryl • oleate • low density lipoprotein receptor • low density lipoprotein receptor-related protein • breath test • apoE • fibroblast • mice

The triacylglycerols present in the lipoproteins transporting lipids from the intestine and from the liver (chylomicrons and very low density lipoproteins) are rapidly hydrolyzed by the enzyme lipoprotein lipase at the endothelial surface of capillaries in adipose tissue, skeletal muscle, cardiac muscle, and other sites (1). The remnant particle resulting from triacylglycerol hydrolysis is then predominantly removed by the liver by a receptor-mediated process, with apoE as the ligand. After endocytosis, the remnant lipoproteins are transported into endosomes and eventually into lysosomes where the lipid components are hydrolyzed.

In previous studies we showed that in rabbits and mice lacking LDL receptors (LDLr), the clearance and metabolism of chylomicron remnants (CR) were markedly decreased as assessed by a breath test which measures the oxidation of fatty acids from CR cholesteryl esters (2, 3). In the absence of the LDLr, other receptors such as the low density lipoprotein receptor-related protein (LRP) may contribute to CR uptake (4–6).

LRP is a multifunctional receptor and has been shown to be identical to the receptor for α_2 macroglobulin (7). Evidence that LRP is an important alternate receptor for CR uptake and metabolism has been provided by Herz and co-workers (4) and Willnow et al. (5, 6). LRP is expressed in many tissues including the liver (8). Inducible tissue-specific inactivation of the LRP gene in the livers of LDL receptor-deficient mice resulted in the accumulation of cholesterol-rich remnants confirming the role of LRP in CR clearance (9).

The receptor-associated protein (RAP), a 39 kDa protein, copurifies with LRP (7) and binds with high affinity to LRP but weakly to the LDL receptor (10, 11). Disruption of the RAP gene resulted in reduced LRP expression in the livers of mice (6). These results are consistent with the proposal that RAP acts as a chaperone for intracellular

Abbreviations: CR, chylomicron remnant; PC, phosphatidylcholine; apoE, apolipoprotein E; CO, cholesteryl oleate; TO, triolein; LDLr, low density lipoprotein receptor; BODIPY-CE, cholesteryl-4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-s-indacene-3-dodecanoate; RAP, receptor-associated protein; PL, phospholipid; FH, familial hypercholesterolemia; GST, glutathione-*S* transferase; SDS, sodium dodecyl sulfate.

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transport of LRP (6). Studies in vitro showed that when RAP was overexpressed as a fusion protein with bacterial glutathione *S*-transferase (GST-RAP) the binding and endocytosis of apoE-enriched β-VLDL in fibroblasts were markedly decreased (10).

In the present study the roles of LDLr and LRP in CR metabolism were assessed in intact mice using a ^{13}C breath test. Breath tests were conducted in LDLr (−/−) knockout, RAP (−/−) knockout, and LDLr(−/−)/RAP(−/−) double knockout mice. In other experiments, GST-RAP was injected to block the LRP receptor activity in vivo. To assess further the role of the binding of remnant-like emulsions to LDL or LRP receptors, experiments were performed with primary cultures of control and LDLr-deficient murine and human fibroblasts. Characteristics of binding of emulsions containing E2, E3, and E4 human apoE isoforms were compared in cells expressing LDL receptors or not expressing LDL receptors. Our findings indicate that while the binding of CR by LDL receptors and by LRP are functionally similar, unless defective the LDL receptors account for the majority of CR uptake.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (PC) was purchased from Lipid Products (Surrey, UK). Cholesterol, cholesteryl oleate (CO), and triolein (TO) were from Nu-Chek-Prep (Elysian, MN). The fluorescent probe, cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-s-indacene-3-dodecanoate (BODIPY-CE) was purchased from Molecular Probes Inc. (Eugene, OR). Suramin was from Bayer (Australia). Lactoferrin was from Sigma (St. Louis, MO).

Animals

Male mice weighing between 20 and 25 g were obtained from the Animal Resources Centre, Murdoch, WA. Mice were obtained at 8 weeks of age and weighed approximately 20 g. Colonies of LDLr (−/−) and LDLr(−/−)/RAP(−/−) nullified by homologous recombination and established from progenitor stocks were obtained from the Jackson Laboratories (Bar Harbor, ME). LDLr heterozygote deficient mice were obtained by cross-breeding C57BL/6J mice with LDLr-deficient mice. ApoB-100 transgenic mice and their controls (Fvb) were obtained from the animal house at the University of California (Berkeley, CA). The presence of the high expressing apoB transgene was associated with a 2.5-fold increase in LDL cholesterol (12).

Preparation of chylomicron-like emulsions

Chylomicron-like emulsions of the required compositions were prepared by sonication and purified by ultracentrifugation as previously described (13). TO, CO, cholesterol, and PC (all from Nu-Chek Prep), each more than 99% pure, were dispensed from stock solutions in $CHCl_3$ into vials. A fluorescent probe (0.1 mg), cholestery 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-s-indacene-3-dodecanoate (CE-BODIPY), purchased from Molecular Probes, was added for preparation of the fluorescently labeled particles. Solvent was removed by lyophilization.

Preparation of remnant-like emulsion particles

Remnant-like emulsions were prepared by sonication and purified by ultracentrifugation as previously described (13). The emulsions were prepared from mixtures of TO (4.5 mg) (PC) (2.5 mg), CO (0.5 mg), and cholesterol (0.8 mg). For preparation of the fluorescently labeled particles, 0.1 mg of a fluorescent probe, BODIPY-CE, purchased from Molecular Probes, was added. After a 1 h sonication of the lipid mixture in 8.5 ml of 2.2% glycerol in water, the crude emulsion was placed at the bottom of two centrifuge tubes, and then 2.5 ml of NaCl solutions of densities 1.065, 1.040, and 1.020 g/ml were sequentially layered above. The tubes were then centrifuged in a SW 41 rotor of a Beckman L8-70M ultracentrifuge for 60 min at 30,000 rpm and 20°C. The particles that floated to the surface were removed and used for cell culture studies.

Sodium dodecyl sulfate (SDS) gel electrophoresis of apoE isoforms

Aliquots of the apoE/remnant-like emulsion mixtures were mixed with 50 μl of reducing buffer (0.17 m Tris-HCl, pH 6.8, 8% SDS, 2 m urea, 40% (v/v) β-mercaptoethanol, and 0.02% (w/v) phenol red). The samples were placed onto a 4% stacking gel that overlayered a 12% sodium dodecyl sulfate polyacrylamide minigel (Hoefer Systems) of 1.5 mm thickness. After staining with Coomassie blue, the gel was imaged using a UMAX scanner.

Iodination of apoE

Human recombinant apoE2, E3, or E4 was iodinated by the lactoperoxidase procedure as previously described (14). Briefly, 0.5 mCi of ^{125}I (as sodium iodide solution) was added to 1 μg of apoE in 3–5 μl of 0.7 m NH_4HCO_3. This was followed by 3 μl of 0.075 m HCl, 2 μl of 0.2 mm H_2O_2, and 2 μl of 1.52 mg/ml lactoperoxidase in 0.05 m citrate buffer solution. After intermittent mixing, a further 2 μl of 0.2 mm H_2O_2 was added followed by intermittent vortexing and addition of 2 μl of 1.52 mg/ml lactoperoxidase in 0.05 m citrate buffer solution. After mixing, 2 μl of 0.05 m NaOH was added to stop the reaction. The mixture was then applied to a desalting column containing Sephadex G-15 (Pharmacia) and washed with 0.7 m NH_4HCO_3 to remove unbound ^{125}I.

Binding of apoE to remnant-like emulsions

Remnant-like emulsions (100 μl) were mixed with 10 μg of human recombinant apoE2, E3, or E4 (Pan Vera Corp., Madison, WI) at a ratio of apoE/TO of 0.13 μg/μg. The emulsion–apoE mixture was sonicated three times for 1 s with a 2 s interval between each sonication pulse using a Sonic and Material Inc. microtip (Danbury, CT).

Cell culture

Primary fibroblast cultures were grown from skin pieces obtained from mice having control C57BL/6J (+/+), LDLr (−/+) and LDLr (−/−) cells. The cells were cultured at 37°C in 1-mL culture dishes (Corning Glass, Corning, NY) containing 1 mL of medium 199 (Flow Lab., Australia) supplemented with 10% fetal calf serum (FCS) (Flow Lab., Australia), plus 2 mm glutamine (Merck), plus antibiotics (100 units/ml penicillin, Calbiochem, San Diego, CA and 100 μg/ml streptomycin, Calbiochem, Marietta, OH) under 5% CO_2/95% air incubator (Hepa Filtered, IR Incubator, Forma Scientific). The medium was renewed twice weekly.

Mouse fibroblast cell line and human HepG2 cells were obtained from ATCC. Control human fibroblast cell lines (GM00) and familial hypercholesterolemia (FH) fibroblast cell lines (GM004680) were obtained from the Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research (Camden, NJ). The cells were cultured at 37°C in 25 mL flask (Corning) containing 5 mL of Eagle's minimal essential medium (MEM) (Flow Lab., Australia) supplemented with 10% fetal calf serum (FCS) (Flow Lab., Australia), plus 2 mm glutamine (Merck), plus

antibiotics (100 units/ml penicillin, Calbiochem, and 100 μg/ml streptomycin, Calbiochem) under 5% CO_2/95% air incubator (Hepa Filtered, IR Incubator, Forma Scientific). Doubling time of the cells was approximately 16 h. The medium was renewed twice weekly. Confluent cultures were subcultured using a split ratio of 1:10. For the binding experiment, stock cells were detached when confluent using 0.025% (w/v) Trypsin/EDTA in Hank's balanced salt solution (BSS), diluted with appropriate media, and plated on coverslips in 35-mm plastic culture dishes. The cover slips were removed from the culture dish and mounted on slides with aquamont for examination by a Bio-Rad MRC-1000 confocal scanning microscope with lens, Plan Apo 60×, NA 1.40.

Binding and uptake studies

In binding studies, cells were preincubated at 37°C for 30 min with 1 ml stock medium 199. Fluorescently labeled remnant-like emulsions (100 μl) containing apoE3 were incubated with primary mouse fibroblast cultures of control C57BL/6J (+/+), LDLr (+/−), and LDLr (−/−) in 1 ml of cold MEM at 4°C for 30 min. For uptake studies, a pulse–chase design was used to study the binding and uptake of fluorescent remnants. An aliquot of fluorescent labeled remnants (100 μl) containing apoE3 was added and incubated for 5 min at 37°C. At the end of the incubation, cells were washed 5 times with BSS (37°C) and then incubated for 30 and 60 min, respectively. The cells were washed 5 times with ice-cold BSS, then fixed and mounted on slides with aquamount.

In other experiments, remnant-like emulsions containing either E2, E3, E4, or mixtures of apoE isoforms (E2/E3, E2/E4, E3/E4) were incubated with primary cultures of either mouse fibroblasts or normal human skin fibroblasts at 4°C for 30 min. After incubation, cells were washed 5 times with cold BSS and then fixed for confocal microscopic examination as described above.

Binding studies with apoE3 were performed with either fluorescent chylomicron-like emulsions or remnant-like emulsions and primary cultures of mouse fibroblasts and various cell lines such as the HepG2, mouse, and human fibrobast cell lines. In other experiments, remnant-like emulsions containing either E2, E3, E4, or 50/50 mixtures of apoE isoforms (E2/E3, E2/E4, E3/E4) were incubated with the cell lines at 4°C for 30 min.

In uptake experiments, remnant-like emulsions containing iodinated apoE2, E3, or E4 were incubated at 37°C for 5 min with control and familial hypercholesterolemia (FH) fibroblasts. Cells were removed from flasks, washed, and then counted for cellular uptake of radioactivity. Similar experiments were conducted with Hep G2 cells.

Assessment of the metabolism of remnants by collection of expired $^{13}CO_2$

A volume of 50 μl of the remnant emulsion mixture was injected via a tail vein into mice. The mice were placed in small chambers for approximately 1.5 min and the exhaled breath was collected into evacuated gas sample containers (Europa Scientific Ltd, Crewe, U.K.). Control C57BL/6J mice and LDLr (−/−) knockout mice were preinjected with either 3 mg of GST or 3 mg of GST-RAP. In other experiments, remnant-like emulsions were injected into C57BL/6J mice, RAP (−/−), LDLr (−/−), and LDLr (−/−)/RAP (−/−) mice. Breath samples were collected at 0 (prior to injection of emulsion) and then at 10, 20, 30, 45, 60, 90, 120, 150, and 180 min after injection of the emulsion. The enrichment of breath samples with $^{13}CO_2$ was measured by isotope-ratio mass spectrometry (ABCA, Europa Scientific, Crewe, U. K.). The $^{13}CO_2/^{12}CO_2$ in the breath samples was compared with a reference standard Peedeebelimnite (PDB_1) and then the delta value was calculated for the sample. The delta value was calculated in units per mil (‰) and has been adopted to express abundance differences as ‰ deviation between two species:

$$\text{Delta}\,^{13}\text{C}(‰) = [(R_{sample}/R_{standard}) - 1] \times 10^3$$

where R is the ratio $^{13}C/^{12}C$.

Preparation of low density lipoproteins

Low density lipoprotein (LDL) was separated from human plasma using a density gradient procedure (15). To 1.8 ml of blood plasma, 685 mg of potassium bromide (KBr) and 45 mg of sucrose were added. The plasma was placed in centrifuge tubes and then overlayered with salt solutions of decreasing density, 1.225 g/ml, 1.1 g/ml, and water. The tubes were centrifuged for 24 h at 4°C and 40,000 rpm. After ultracentrifugation, the LDL band (yellow layer) was carefully removed from the tube. Phosphate-buffered saline (PBS), pH 7.4, was then used to dialyze the LDL at 4°C overnight. The LDL was then assayed for protein content.

Chemical analysis

Lipid phosphorus was measured by the method of Bartlett (16). The lipids extracted from remnants with chloroform–methanol 2:1 (vol/vol) were separated by thin-layer chromatography. Triglyceride (TG) was quantified as glycerol by chromotropic acid (17), free and esterified cholesterol were assayed by the *o*-phthaldialdehyde procedure (18) after saponification of lipids in the separated bands. Protein was assayed by the procedure of Lowry et al. (19).

LDLr genotyping of mice by polymerase chain reaction (PCR) technique

The LDLr genotyping of C57BL/6J (+/+), LDLr (+/−), and LDLr (−/−) mice was performed using PCR techniques as described previously (6). A biopsy from each mouse was taken from the tail. The DNA was extracted from the tail, separated by gel electrophoresis, and DNA bands were visualized for genotyping.

Statistical analysis

The data were analyzed using a 1-way analysis of variance (AOV) to establish significant differences among groups. A 2-tailed *t*-test was used to compare differences between means.

RESULTS

Characterization of apoE/remnant-like emulsion mixtures

In **Fig. 1**, the remnant-like emulsion particles were of average diameter 73 ± 7 nm (n = 30) as measured by negative-stain electron microscopy. The composition of remnant-like emulsion was (% by mass, n = 3) TO, 57.1 ± 1.6, CO, 8.1 ± 0.9, cholesterol, 7.7 ± 0.8, and PC, 27.1 ± 2.0. **Figure 2** shows the apoE content (E2, E3, E4) of remnant-like emulsion particles on SDS polyacrylamide gel electrophoresis.

Effect of apoE isoforms on the binding of fluorescently labeled remnant-like emulsions to primary cultures of mouse and human fibroblasts

The binding of remnant-like emulsions containing apoE2, E3, or E4 emulsions (apoE/TG of 0.13 μg/μg) to mouse fibroblasts was compared in **Fig. 3** (upper panel). Remnant-like emulsions containing apoE2 were found to bind poorly to either control C57BL/6J (+/+), LDLr

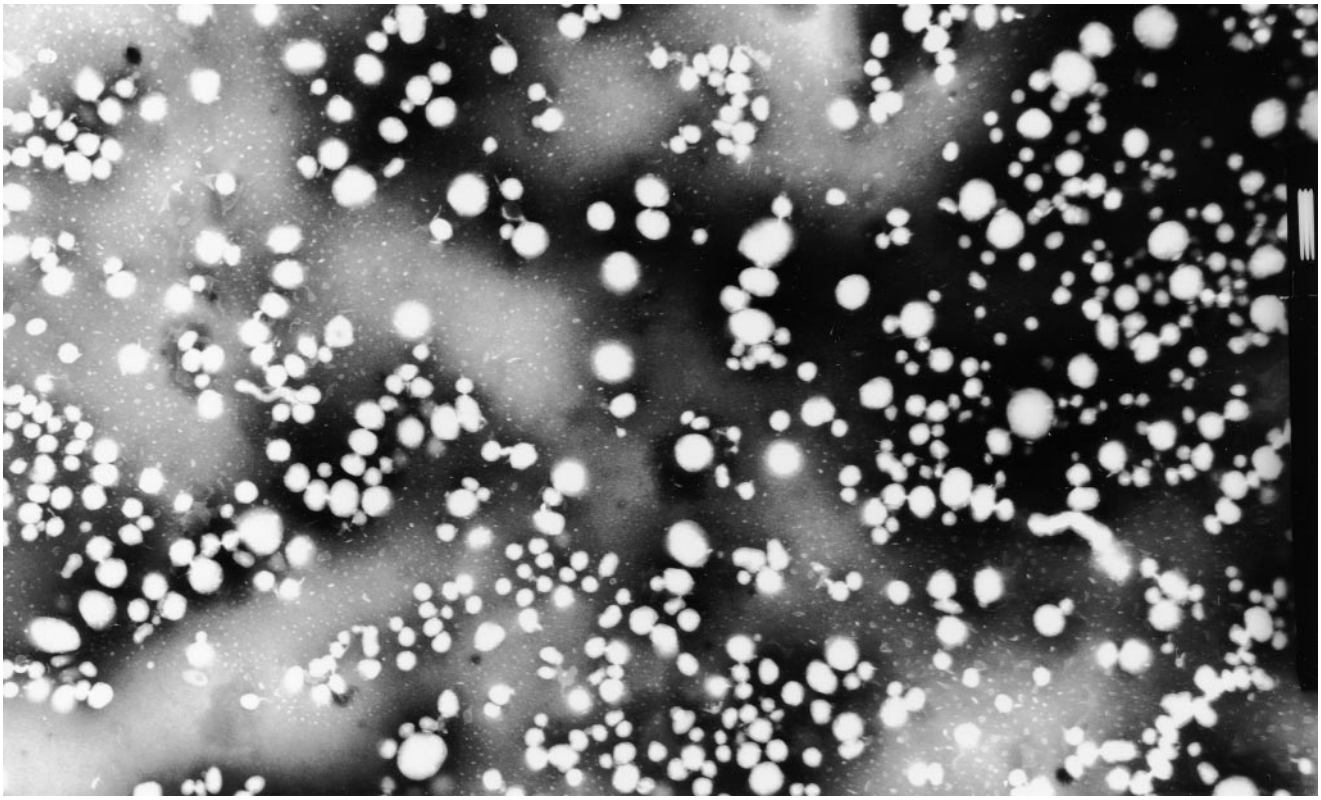

Fig. 1. Electronmicrograph of remnant-like emulsions. The bar represents 200 nm.

(−/+) or LDLr, (−/−) mouse fibroblasts. In the presence of either apoE3 or apoE4, the cell-associated fluorescence was markedly increased when compared with the binding of apoE2 to mouse fibroblasts. In Fig. 3 (lower panel) with human fibroblasts in the presence of apoE2, the binding of remnant-like emulsions was also markedly lowered as shown by the minimal fluorescence when compared with apoE3 or apoE4 isoforms. The binding to human fibroblasts of remnant-like emulsions containing a mixture of apoE isoforms (E3/E4) was similar when compared with the binding of emulsions containing only apoE3 or only apoE4. With mixtures containing apoE2

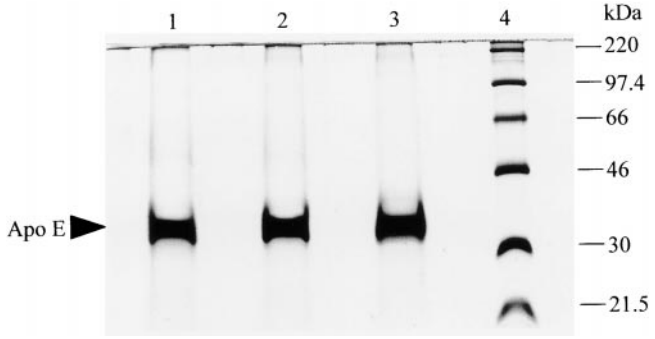

Fig. 2. The apoE isoforms (10 μg) associated with remnant-like emulsions applied to a vertical slab of SDS polyacrylamide gel. Lanes 1, 2, and 3 contain emulsion mixtures with apoE2, apoE3, and apoE4, respectively. Lane 4 contains molecular weight markers.

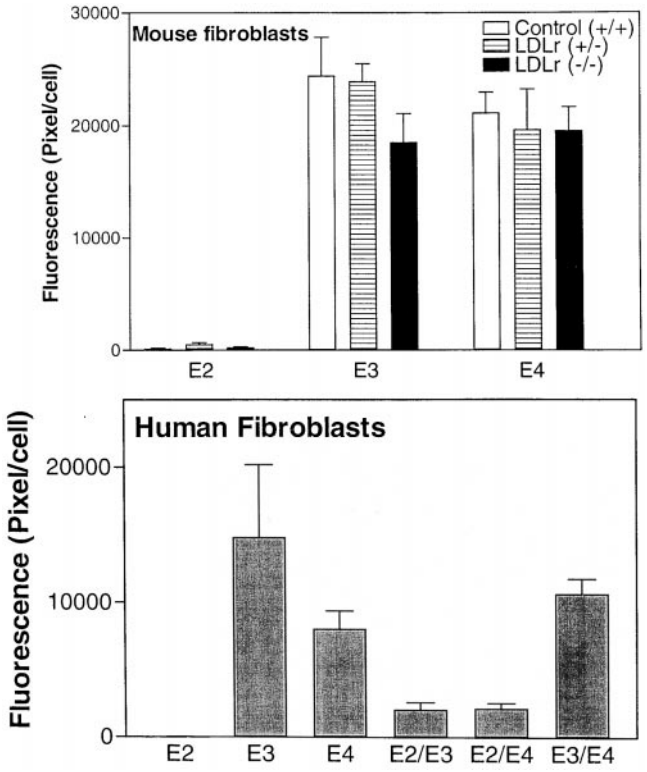

Fig. 3. The effect of apoE isoforms on the binding of remnant-like emulsions to mouse and human fibroblasts at 4°C. One hundred μl of fluorescent emulsions was added to the incubations in 1 ml of cold MEM at 4°C for 30 min. Results are means ± SEM of four experiments in each group.

(E2/3 or E2/4) the binding to remnant-like emulsions was markedly decreased when compared with emulsions containing either apoE3, E4, or E3/E4. Similar results were obtained for the binding of fluorescent emulsions containing apoE isoforms to human HepG2 and mouse fibroblast cell lines (data not shown). The concentration of apoE per 100 μl of emulsion was at a ratio of apoE/TO of 0.13 μg/μg.

Binding of fluorescent chylomicron and remnant-like emulsion to mouse fibroblasts and HepG2 cells

In **Fig. 4**, the binding of fluorescent chylomicron-like emulsions (A) to control C57BL/6J fibroblasts was virtually negligible when compared with the binding of fluorescent remnant-like emulsions (B). In Fig. 4, chylomicron-like emulsions (C) bound poorly to HepG2 cells when compared with remnant-like emulsions (D). The content of apoE3 per 100 μl of emulsion was at a ratio of apoE3/TO of 0.13 μg/μg.

Binding of remnant-like particles to control C57BL/6J (+/+), LDLr (−/+), and LDLr(−/−) mouse fibroblasts

In **Fig. 5** (upper panel), the binding at 4°C of remnant-like emulsions containing apoE3 to control C57BL/6J (+/+), LDLr heterozygous (−/+), and LDLr homozygous (−/−) fibroblasts was compared. Skin fibroblasts from LDLr (−/−) and LDLr (−/+) cells were shown to bind similar quantities of remnant-like emulsions as indicated by similar cell-associated fluorescence when compared with control (+/+) cells. In the absence of apoE3, there was no cell-associated fluorescence in fibroblast cells, indicating that there was no binding of remnant-like emulsions to fibroblast cells (data not shown).

Figure 5 (lower panel) shows that markedly higher fluorescence was associated with LDLr (−/−) and LDLr (+/−) fibroblast cells at 30 min ($P < 0.001$) when compared with control cells incubated at 37°C. By 60 min, markedly less ($P < 0.002$) fluorescence was associated with control and receptor-deficient fibroblasts when compared with studies at 30 min, indicating the metabolism and degradation of fluorescent remnants with time. The cell-associated fluorescence at 60 min was greater for LDLr (−/+) and LDLr (−/−) cells when compared with control fibroblasts.

Binding of remnant-like emulsion particles to human fibroblasts

In **Fig. 6** the cell-associated fluorescence (incubation at 4°C) was similar in control fibroblasts and FH fibroblasts, indicating that the binding of remnant-like emulsions containing apoE3 was normal in FH cells. When fluorescent emulsions contained apoE2, there was virtually no binding to either control or FH fibroblasts when compared with emulsions containing apoE3 or apoE4.

Uptake of ^{125}I-labeled apo E by human fibroblasts

In **Fig. 7** the uptake (incubation at 37°C) by control and FH fibroblasts of ^{125}I-labeled apoE2 labeled non-fluorescent remnant-like emulsions was markedly less when compared with remnant-like emulsions containing either radiola-

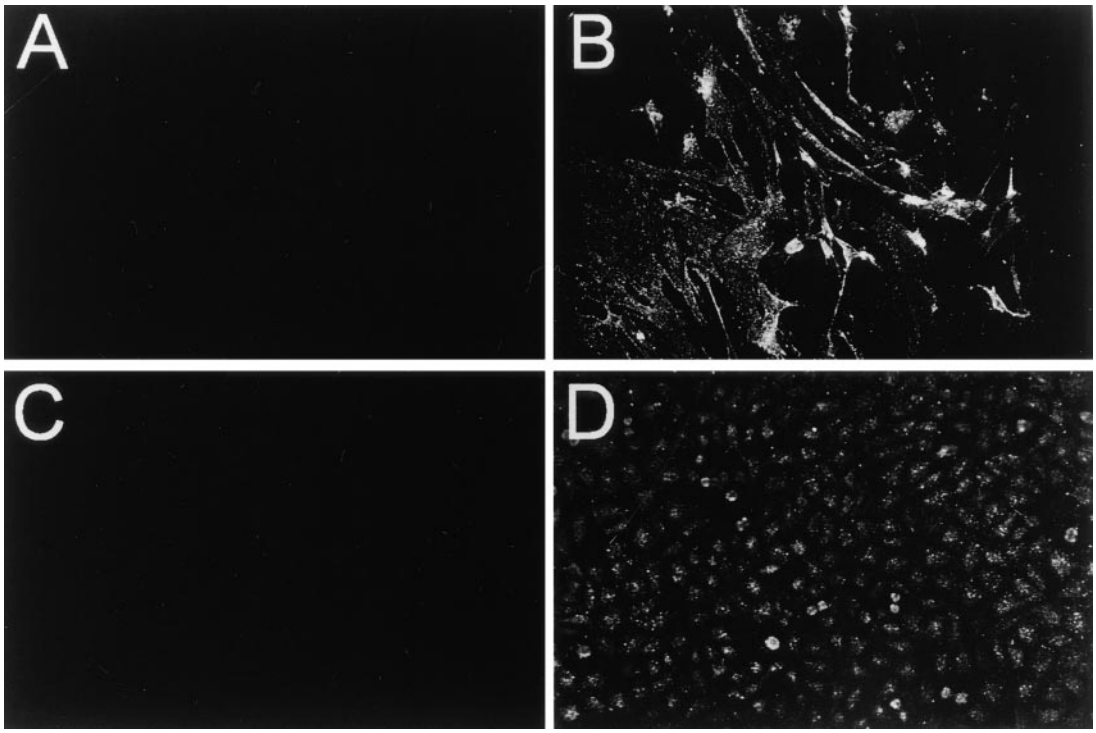

Fig. 4. The binding of fluorescent chylomicron-like and remnant-like emulsions to mouse fibroblasts and HepG2 cells. Control mouse fibroblast cells were incubated with chylomicron-like emulsions (A) and remnant-like emulsions (B). HepG2 cells were incubated with chylomicron-like emulsions (C) and remnant-like emulsions (D). One hundred μl of fluorescent emulsions containing apoE3 was added to the incubations in 1 ml of cold MEM at 4°C for 30 min.

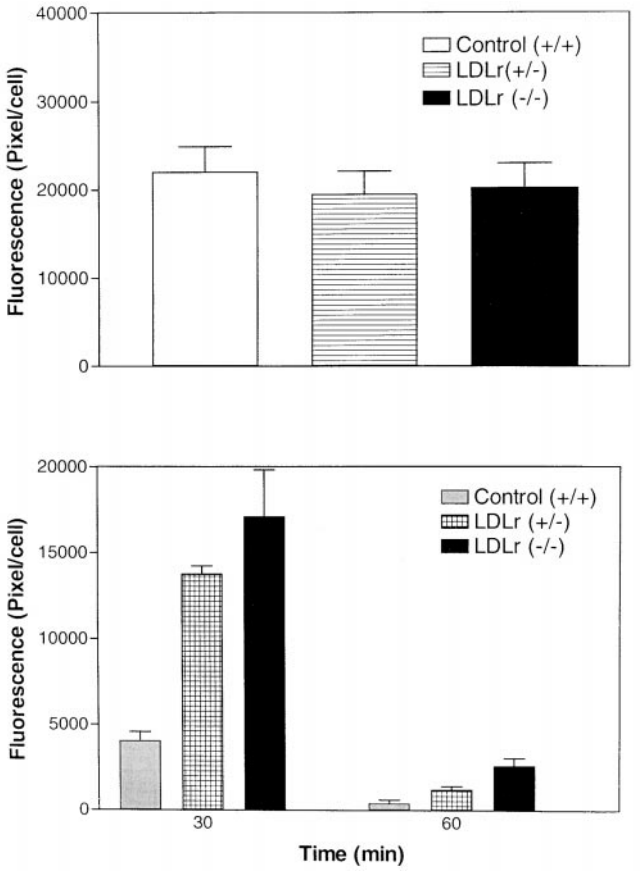

Fig. 5. The binding and metabolism at 37°C of fluorescent remnant-like emulsions containing apoE3 by skin fibroblasts obtained from control C57BL/6J (+/+), LDLr (+/−) or LDLr (−/−) mice. Results are means ± SEM of 8 experiments in each group.

beled apoE3 or apoE4. The uptake of radiolabeled apoE3 or apoE4 was less with FH fibroblasts when compared with control fibroblasts ($P < 0.001$). In other experiments with HepG2 cells, the uptake of ^{125}I-labeled apoE2 was less when compared with non-fluorescent emulsions containing radiolabeled apoE3 or apoE4 (data not shown).

Effect of human low density lipoprotein (LDL) on the binding of remnant-like emulsions to fibroblasts

Competition by LDL for the binding of remnant-like emulsion to fibroblasts in culture is illustrated in **Fig. 8**.

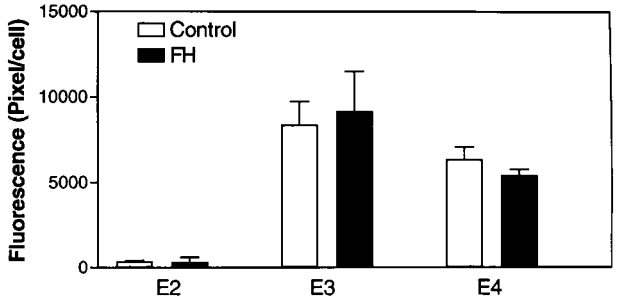

Fig. 6. The binding at 4°C of fluorescent remnant-like emulsions by normal control and FH fibroblast cells. The results are means ± SEM of 4 experiments in each group.

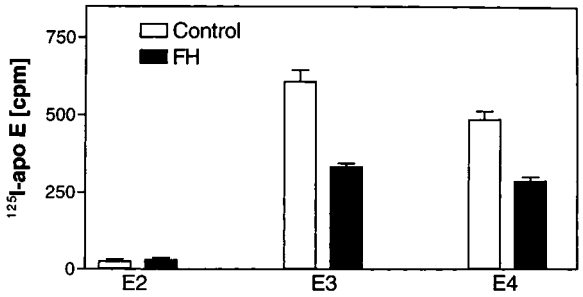

Fig. 7. The uptake at 37°C of ^{125}I-labeled apoE containing non-fluorescent remnant-like emulsions by control and FH fibroblasts. The results are means ± SEM of 4 experiments in each group.

The presence of LDL at a protein concentration of 10 μg/ml decreased the cell-associated fluorescence, indicating that the binding of remnant-like emulsions to the control (+/+) cells was reduced. Increasing doses of LDL resulted in a dose-dependent decrease ($P < 0.001$) in cell-associated fluorescence (Fig. 8).

In the LDLr (+/−) cells, there was no significant effect of either 10 or 50 μg of LDL on the binding of remnant-like emulsion. However, at a concentration of 100 μg/ml of human LDL, the binding of remnant-like emulsions to cells was markedly decreased ($P < 0.001$). In LDLr (−/−) cells, a decrease ($P < 0.001$ by AOV) in cell-associated fluorescence was found at all doses of LDL when compared with incubations without LDL.

Effects of lactoferrin or suramin on the binding of fluorescent labeled remnant-like emulsions to fibroblasts

Figure 9 shows the effect of lactoferrin and suramin on the fluorescence associated with control (+/+), LDLr (+/−), and LDLr (−/−) fibroblasts when in culture with remnants containing apoE3 at 4°C. The upper panel shows lactoferrin at concentrations of 0.5 mg/ml or greater completely inhibited the binding of the fluorescent CR in all fibroblast cell types. Also shown in Fig. 9 (lower panel), suramin also markedly inhibited the binding of remnant-like emulsions to all cell types at concentrations of 0.25 mg/ml or higher.

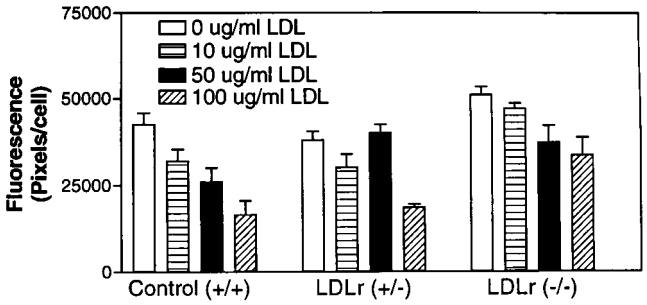

Fig. 8. The effect of human LDL on the binding at 4°C of fluorescent remnant-like emulsions to control and LDLr-deficient mouse skin fibroblasts. The binding of remnant-like emulsions to cells was assessed at doses of LDL ranging between 10 and 100 μg/ml. The results are means ± SEM of 4 experiments in each group.

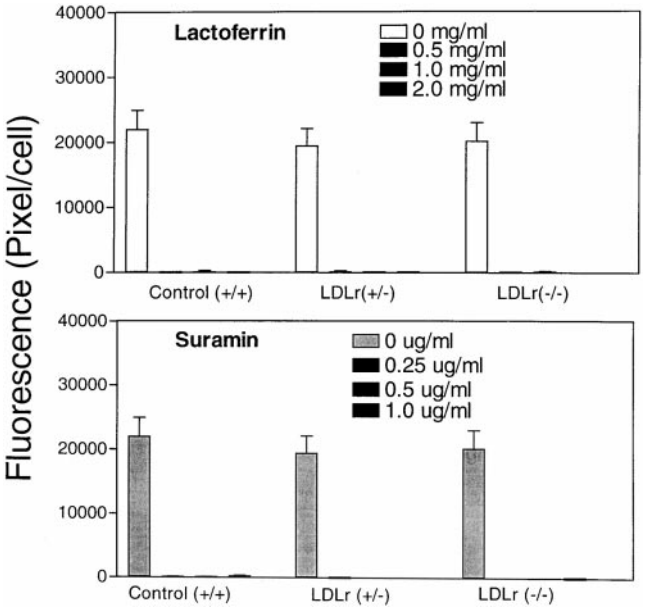

Fig. 9. The effects of lactoferrin and suramin on the binding at 4°C of fluorescent remnant-like emulsions to mouse skin fibroblasts. The results are means ± SEM of 4 experiments in each group.

Measurement of remnant metabolism from CO_2 in expired breath of C57BL/6J mice or mice homozygous for deficiencies in LDLr (−/−), RAP (−/−), or LDLr (−/−)/RAP (−/−)

Figure 10 compares the enrichment of $^{13}CO_2$ in the expired breath of control C57BL/6J mice, homozygous LDLr-deficient, homozygous RAP (−/−), and homozygous LDLr(−/−)/RAP(−/−) mice after injection of emulsions containing cholesteryl[^{13}C]oleate. Compared with the marked enrichment with ^{13}C peaking at 45 min in control C57BL/6J mice, in LDLr(−/−)/RAP(−/−)-deficient mice there was very little change in the enrichment of $^{13}CO_2$ in the expired breath. In LDLr-deficient mice the rate of expiration of $^{13}CO_2$ in expired breath was more than in the double-knockout mice but significantly less ($P < 0.001$) when compared with either control or RAP (−/−) knockout mice as calculated by AUC over the first 45 min. In RAP (−/−) knockout mice, the breath enrichment was appreciable but still significantly less ($P < 0.002$) than in control C57BL/6J mice.

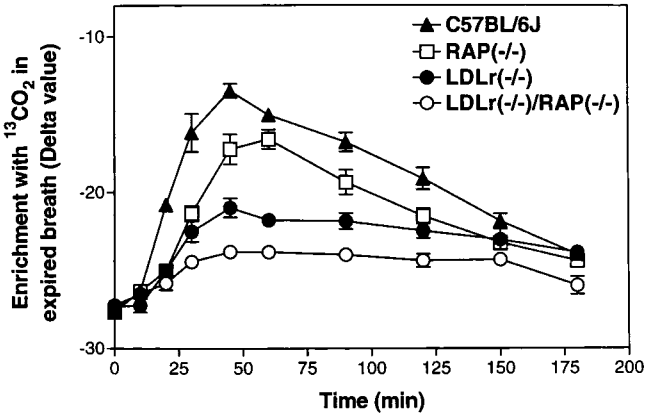

Fig. 10. The enrichment of $^{13}CO_2$ in the expired breath of control C57BL/6J, RAP (−/−), LDLr (−/−) and LDLr(−/−)/RAP (−/−) mice after injection of remnant-like emulsions containing cholesteryl[^{13}C]oleate. Mice were injected with 50 μl of remnant-like emulsions containing cholesteryl [^{13}C]oleate via the tail vein and expired breath was collected as described under Methods. The results are means ± SEM of 4 animals in each group.

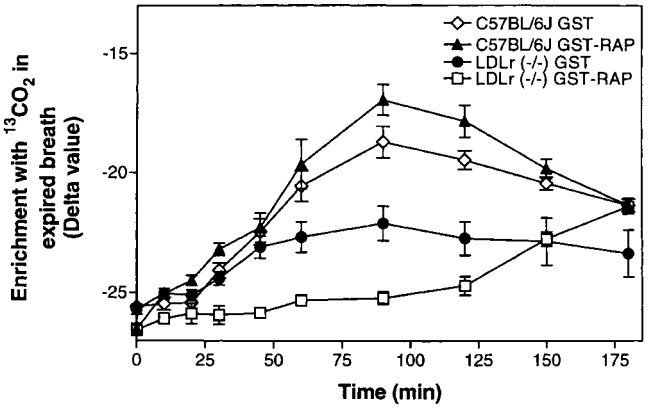

Fig. 11. The enrichment of $^{13}CO_2$ in the expired breath of control C57BL/6J mice and LDLr (−/−) knockout mice preinjected with either 3 mg of GST or GST-RAP after injection with remnant-like emulsions containing cholesteryl[^{13}C]oleate. The results are means ± SEM of 4 animals in each group.

In **Fig. 11** the patterns of enrichment of $^{13}CO_2$ in the expired breath of control C57BL/6J and LDLr (−/−) mice were compared after pre-injection of either 3 mg of GST, or GST-RAP. In control C57BL/6J mice pre-injected with GST, the enrichment of $^{13}CO_2$ in the expired breath was found to increase to a peak at approximately 90 min and then decline after injection of emulsions containing cholesteryl[^{13}C]oleate. When injected with GST-RAP, the peak was similar; in fact, the breath enrichment of $^{13}CO_2$ in C57BL/6J mice injected with GST-RAP was slightly increased compared with mice injected with GST alone. Consistent with previous findings (2, 3) in LDLr knockout mice, the breath enrichment was less ($P < 0.01$) when compared with control C57BL/6J when both received GST alike (Fig. 11). When LDLr knockout mice were injected with GST-RAP, breath enrichment was further suppressed when compared with LDLr knockout mice injected with GST alone.

Effect of expression of human apoB-100

In previous studies Callow et al. (12) found that plasma cholesterol levels were elevated in apoB-100 transgenic mice. The rise in plasma cholesterol in apoB-100 transgenic mice resulted from the increase (2.5-fold) in human-like LDL particles. Therefore, this was a convenient model to test the hypothesis that elevated LDL concentrations in vivo would compete for the clearance of CR. In **Fig. 12** the enrichment of $^{13}CO_2$ in the expired breath of apoB transgenic mice was significantly decreased ($P < 0.05$) when

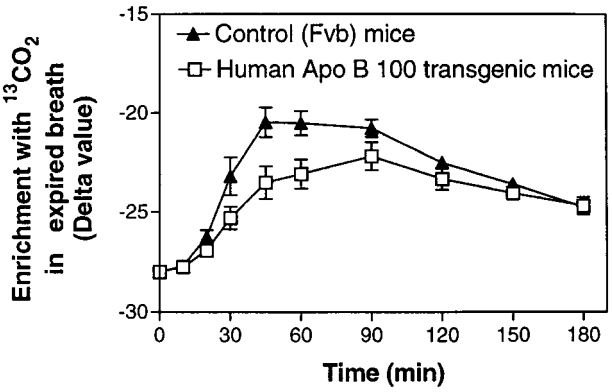

Fig. 12. The appearance of $^{13}CO_2$ in the expired breath of control FvB mice and human apoB-100 transgenic mice injected with remnant-like emulsions. The results are means ± SEM of 4 animals in each group.

compared with controls as calculated by the AUC over the first 60 min.

DISCUSSION

Current evidence for the uptake and metabolism of CR indicates a two-receptor model in which both the LDLr and LRP receptors can mediate the binding and endocytosis of CR (11). In the present study the relative contribution of each of these receptors has been assessed. We previously found that the metabolism of CR in the livers of mice deficient in LDLr was delayed and that the metabolism of remnants was mediated by an alternative apoE-dependent pathway, probably via LRP (20). In the present study the metabolism of remnant-like emulsions was compared in LDLr-deficient, RAP-deficient, and LDLr/RAP-deficient mice using a newly developed ^{13}C breath test (3). The specificity and sensitivity of the ^{13}C breath test in detecting changes in CR metabolism has been previously assessed with different ^{13}C-labeled cholesteryl esters, various transgenic mice, and effects of feeding and diurnal variation (3). The results from these studies also showed that the metabolism of fatty acids was unaffected and not related to defects in CR metabolism found in LDLr-deficient, apoE-deficient mice, and diabetic rats (2,3). Lipid remnant-like emulsions provide advantages because they lack exogenous proteins or apolipoproteins which complicate interpretations. We have also measured in this study the binding and endocytosis of fluorescent remnant-like emulsions by skin fibroblasts obtained from control C57BL/6J mice and from mice lacking the LDLr. The binding of remnant-like emulsions containing the human apoE isoforms and mixtures of these isoforms were compared in primary cultures of mouse and human skin fibroblasts.

In intact mice the enrichment of $^{13}CO_2$ in the expired breath was markedly decreased in LDLr/RAP-deficient mice indicating that the metabolism of remnant-like emulsions was essentially nullified in the absence of both LDLr and LRP receptors (Fig. 10). In RAP knockout mice, the breath enrichment was significantly decreased consistent with the role of LRP receptors in the metabolism of CR (Fig. 10). To confirm further the role of LRP in remnant metabolism, LDLr mice were pre-injected with GST-RAP, which caused a marked delay in the appearance of $^{13}CO_2$ in the expired breath of homozygous LDLr deficient mice when compared with their respective controls (Fig. 11).

In recent studies gene inactivation of LRP in the liver was found to impair the clearance of CR (9). In our studies the contribution to binding of CR by another receptor was confirmed in skin fibroblasts from LDLr-deficient mice, which showed similar binding of fluorescent remnant-like emulsions when compared with fibroblasts from control mice (Fig. 5, upper panel). The binding of fluorescent emulsions was also found to be similar between control human fibroblasts and fibroblasts from an FH individual (Fig. 6). However, the uptake of labeled apoE-containing emulsions was found to be markedly less in FH fibroblasts (Fig. 7). Furthermore, although binding at 4°C of remnant-like emulsions was unimpaired in fibroblasts from LDLr-deficient mice, an effect on endocytosis and subsequent metabolism could not be ruled out because at 37°C in a pulse–chase design the disappearance of fluorescence was markedly slower when compared with control fibroblasts (Fig. 5, lower panel). These findings suggest that another receptor, probably LRP, plays an important role in the binding of CR in LDLr-deficient fibroblasts.

In mouse fibroblast cells, human LDL inhibited the binding of fluorescent remnant-like emulsions. In fibroblasts from control mice incubated with increasing amounts of LDL, there was a dose-dependent decrease in the binding of fluorescent remnant-like emulsions (Fig. 8). In LDLr-deficient cells, a small but significant decrease in remnant binding was also found, indicating that LDL may bind to LRP and compete for the binding of fluorescent remnant emulsions. To assess the in vivo significance of competition by LDL for CR metabolism, breath tests were performed in mice expressing human apoB-100. Callow et al. (12) have previously shown that apoB-100 transgenic mice have elevated plasma cholesterol levels (2.5-fold) as a result of the increase in human-like LDL particles. The metabolism of emulsion remnants in the apoB-100 transgenic mice was markedly decreased as assessed by the ^{13}C breath test (Fig. 12). These findings suggest that LDL compete in vivo with CR for clearance via the LDL and LRP receptors.

ApoE3 and apoE4 but not apoE2 have been shown to bind strongly to the LDL receptor (21). Our studies establish for the first time the similar characteristics of binding of apoE isoforms by LDLr and by LRP receptors. In our studies, fluorescent or non-fluorescent remnant-like emulsions containing human apoE2 did not bind to either mouse or human fibroblasts (Figs. 3 and 6, 7), whereas emulsions containing human apoE3 orE4 bound avidly to the cells whether or not expressing LDLr. With mixtures of apoE isoforms containing apoE2 (E2/E3 or E2/E4) the binding to human fibroblast cells was markedly decreased compared with E3 or E4 alone (Fig. 3, lower panel). Fibroblasts incubated with the emulsions containing the apoE3/

E4 isoform mixture showed cell-associated fluorescence similar to those with only apoE3 in the remnant-like emulsion.

The binding of lipoproteins appears to involve charge interactions between cysteine-rich domains in the LDL receptor and clusters of basic amino acids in apoE (21). The charged glycoprotein lactoferrin has been shown in vivo to inhibit the clearance of CR in control and LDLr-deficient mice (20). Similarly, in intact rats, the clearance of CR was markedly decreased by lactoferrin (22). Suramin is a polyanionic, highly sulfated compound which also inhibits the clearance of CR (20). In the present studies when suramin and lactoferrin were incubated with control or LDLr-deficient cells, there was virtually no cell-associated fluorescence consistent with the in vivo data and again showing the similarity of binding of CR by LDLr and LRP (Fig. 9).

Our studies show that the LDL receptor under normal physiological circumstances provides the major pathway for CR clearance but LRP provides an alternate potential pathway. The competition of CR with LDL for a common removal pathway via the LDL receptor was established by a marked delay in emulsion remnant metabolism in apoB-100 transgenic mice. When LDLr are deficient as in LDLr knockout mice, our results show that LRP becomes the major pathway for remnant metabolism and the metabolism is completely abolished by GST-RAP. In vitro studies with fibroblasts provide further evidence that another receptor, probably LRP, accounts for binding of emulsions to the LDLr-deficient cells. Whether mediated by LDLr or LRP, human apoE3 or E4 were essential for binding of emulsions to cells but not apoE2. We conclude that under normal physiological conditions LDLr are important for the metabolism of CR and LRP plays a minor role. However, when LDLr are defective, the LRP receptor is able to take over and become the major pathway for uptake and metabolism of cholesterol-rich remnants by the liver. This adaptive response may involve up-regulation of expression of LRP but details remain to be elucidated.

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