Uptake and catabolism of modified LDL in scavenger-receptor class A type I/II knock-out mice

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The liver is the major organ responsible for the uptake of modified low-density lipoprotein (LDL) from the blood circulation, with endothelial and Kupffer cells as major cellular uptake sites. Scavenger-receptors, which include various classes, are held responsible for this uptake. Mice deficient in scavenger-receptor class A types I and II were created and the fate of acetylated LDL (Ac-LDL) *in vivo* and its interaction with liver endothelial, Kupffer and peritoneal macrophages was characterized. Surprisingly, the decay *in vivo* ($t_{\frac{1}{2}} < 2$ min), tissue distribution and liver uptake (at 5 min it was $77.4 \pm 4.6\,\%$ of the injected dose) of Ac-LDL in the knock-out mice were not significantly different from control mice ($t_{\frac{1}{2}} < 2$ min and liver uptake $79.1 \pm 4.6\,\%$ of the injected dose). A separation of mice liver cells into parenchymal, endothelial and Kupffer cells 10 min after injection of Ac-LDL indicated that in both control and knock-out mice the liver endothelial cells were responsible for more than 70 % of the liver uptake. Both in control and knock-out mice, preinjection of polyinosinic acid (poly I, 200 μg) completely blocked the liver uptake, indicating that both in control and knock-out mice the scavenger-receptors are sensitive to poly I. Preinjection of suboptimal poly I concentrations (20 and 50 μg) provided evidence that the serum decay and liver uptake of Ac-LDL is more readily inhibited in the knock-out mice as compared with the control mice, indicating less efficient removal of Ac-LDL *in vivo* in the knock-out mice under these conditions. Studies *in vitro* with isolated liver endothelial and Kupffer cells from knock-out mice indicate that the cell association of Ac-LDL during 2 h at 37 °C is 50 and 53 % of the control, respectively, whereas the degradation reaches values of 58 and 63 %. For peritoneal macrophages from knock-out mice the cell association of Ac-LDL was identical to the control mice whereas the Ac-LDL degradation in cells from the knock-out mice was 17 % of the control. The low degradation capacity of peritoneal macrophages from knock-out mice for Ac-LDL indicates that scavenger-receptor class A types I and II play a quantitative important role in the degradation of Ac-LDL by macrophages. In liver, the contribution of scavenger-receptor class A types I and II to the maximal uptake and degradation of Ac-LDL by endothelial and Kupffer cells was 40–50 %. Binding studies performed at 4 °C indicate that the lower rates of degradation are due to a lower number of surface receptors on the cells from the knock-out mice. From the *in vitro* and *in vivo* data it can be concluded that in addition to the classic scavenger-receptors class A types I and II liver does contain additional novel poly I-sensitive scavenger-receptors that facilitate efficient removal of Ac-LDL from the blood circulation. The availability of the scavenger-receptor class A types I and II knock-out mice will stimulate further molecular identification of these receptors.

INTRODUCTION

Macrophage-derived foam cells are an important feature of the early atherosclerotic lesions [1]. Native low-density lipoprotein (LDL) does not provoke foam-cell formation because effective accumulation of cholesterol (esters) is prevented by down-regulation of the native LDL receptor [2]. Various modifications of LDL, including acetylation [3], acetoacetylation [4], malondialdehyde treatment [5] and oxidation [6], lead to scavenger-receptor-mediated uptake by macrophages, coupled with a lipid accumulation with similar characteristics as observed in the atherosclerotic plaque [7].

Some years ago it was established that, when injected *in vivo*, acetylated LDL (Ac-LDL) is rapidly cleared from the blood by the liver [8]. The Ac-LDL receptor was partially purified from total liver with an estimated molecular mass of 220–250 kDa [9]. Kodama et al. [10] purified a protein with a molecular mass of 220 kDa from bovine lung membranes. This protein was subsequently cloned and it appeared that two scavenger-receptor isoforms, called type I and type II, are generated by alternative splicing of a message encoded by a single gene [11,12]. Expression of both types of scavenger receptor has been detected in macrophages *in vitro* [11,13] and *in vivo* [14,15]. The major sites of expression *in vivo* are tissue macrophages, although liver endothelial cells are also immunostained with the monoclonal antibody 2F8 [16]. The recent identification of additional scavenger-receptors such as Marco [17], macrosialin [18,19], CD 36 [20] and scavenger-receptor B1 [21] has posed questions relating to their relative role and specificity in modified-LDL recognition and uptake in the body.

The rapid clearance of Ac-LDL by the liver is mainly exerted by liver endothelial cells [22] while oxidized LDL (Ox-LDL) is concentrated in Kupffer cells [23]. As an extension of studies with mouse peritoneal macrophages we assumed that Ac-LDL uptake is exerted by scavenger-receptors class A types I and II, while a specific binding protein for Ox-LDL in Kupffer cells was observed [23,24] which was recently identified as macrosialin [19]. The generation of mice deficient in scavenger-receptor class A types I and II [25] enables us to test whether these receptors are indeed mainly responsible for the clearance and metabolism of Ac-LDL. In addition, the potential importance of other modified-LDL receptors might become clear from such a model.

Abbreviations used: LDL, low-density lipoprotein; Ox-LDL, oxidized low-density lipoprotein; Ac-LDL, acetylated low-density lipoprotein; poly I, polyinosinic acid; TCA, trichloroacetic acid; ASOR, asialo-orosomucoid.

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MATERIALS AND METHODS

Chemicals

Nycodenz was obtained from Nycomed A/S, Oslo, Norway. Collagenase type I and BSA (fraction V, defatted) were purchased from Sigma; ^{125}I (carrier-free) was from New England Nuclear Chemicals, Dreieich, Germany; Ham's F-10 medium was from Gibco-Europe, Hoofddorp, The Netherlands.

LDL, isolation, iodination, oxidation and acetylation

LDL was isolated from human plasma plus 1 mM EDTA, at a density of 1.024–1.055, by two repetitive centrifugations according to [26] as described in [27]. The LDL preparation contained mostly apolipoprotein B (99.97 %) and no degradation products were noticeable when checked by electrophoresis in SDS gels. With a high LDL concentration (5 mg of apolipoprotein/ml) in a radial immunodiffusion system according to [28], apolipoprotein E was noticeable at the detection limit and contributed at most 0.02–0.03 % to the total apolipoprotein. Radio-iodination of LDL was carried out according to a modification [29] of the ICl method [30]. Radio-iodination and coupling of tyramine-cellobiose to Ac-LDL were carried out as described previously [31,32]. LDL was acetylated with repeated additions of acetic anhydride according to Basu et al. [33] as described previously in detail [8].

Transgenic mice

Mice deficient in type I and type II class A scavenger-receptors were generated by disrupting exon 4 of the gene, which is essential for the formation of functional trimeric receptors [25]. Then 3-1 embryonic stem cells were transfected by electroporation and such cells containing the disrupted allele were injected into C57Bl/6J blastocytes. Embryos were transferred into the uteri of ICR recipients. To obtain hereozygous mutants, chimaeras were mated with ICR females. Brother–sister mating of heterozygotes was used to generate homozygous mutants (−/−) and controls (+/+), which were thus littermates. Mice heterozygous and homozygous for the mutation were normal in appearance, growth and fertility. Deficiency was transmitted in a Mendelian fashion, as identified by Southern-blot analysis of tail DNA. Immunostaining of liver sections from wild-type animals, using monoclonal antibody 2F8 [16], detected class A scavenger-receptors in liver endothelial and Kupffer cells while in homozygous receptor-deficient mice no immunoreactive protein was detected [25]. 2F8 can bind to both type I and type II class A scavenger-receptors, indicating the homozygous receptor-deficient mice are deficient in both types I and II receptor protein.

Serum clearance *in vivo* and liver association

Throughout this study 6–7-week-old mice were used which were anaesthetized by intraperitoneal injection of 2.1 mg of Nembutal. The abdomens was opened, and radiolabelled compounds were injected into the inferior vena cava at the level of the renal veins [34]. The body temperature of the mice was maintained at 36.5–37 °C by an infrared heating lamp. At the indicated times, 50 μl of blood was taken from the inferior vena cava at least 0.5 cm distal of the injection point. The samples were centrifuged for 2 min at 20000 ***g***, and the radioactivity in the supernatants was counted. Liver lobules were tied off and excised at the indicated times. After weighing the lobule and counting its radioactivity, the total liver uptake was calculated using the assumption that 4.8 % of the total body weight is contributed by the liver. The amount of liver that was tied off was 2–3 % at each time point, so that at the longest circulation time still less than 15 % of the total liver was removed. The percentage-of-liver value was corrected for the amount of sample present in the entrapped blood based upon ^{3}H-labelled albumin measurements. When indicated, polyinosinic acid (poly I) was injected as a bolus at 1 min prior to the radiolabelled compound.

Cell isolation procedures

Mice were anaesthetized and injected with the radiolabelled compounds in a similar way as for the determination of the total liver uptake. At 10 min after injection the heart aorta was cannulated, and a liver perfusion was started with Hank's buffer, pH 7.4, plus Hepes (1.6 g/l) at 37 °C. After 10 min perfusion (flow rate, 14 ml/min) a lobule was tied off for determination of the total liver uptake. To separate the various cell types, the liver was subsequently subjected to perfusion with inclusion of 0.025 % (w/v) collagenase. The separation of parenchymal cells was performed as described earlier for rats [35].

Endothelial and Kupffer cells were purified by centrifugal elutriation exactly as described earlier for rats [22,35]. Purity of the cells was assessed by phase-contrast microscopy. The purity of the endothelial cell preparation was higher than 90 %, whereas Kupffer cell preparations contained 70–90 % Kupffer cells, the remaining being endothelial cells. The microscopical purity determinations were controlled by assessing the uptake of latex (size 0.8 μm) by Kupffer cells, while endothelial cells are negative. Calculation of the contribution of the different cell types to total liver uptake was performed as described [8]. A quantitative recovery of the radioactivity associated with total liver in the subsequently isolated cells was obtained by using the tyramine-cellobiose-labelled Ac-LDL. This was checked for each individual cell isolation by comparing the calculated (from the relative contribution of the various cell types) and determined total liver association values. Peritoneal macrophages (unstimulated) were collected from animals of each genotype by lavage of the peritoneum with 10 ml PBS/1 mM EDTA. Cells were centrifuged for 10 min at 750 g and subsequently purified on Nycodenz [22,35].

Cell binding, association and degradation of lipoproteins *in vitro*

Parenchymal, Kupffer and endothelial cells were isolated by the same procedure as used for determination of the uptake of lipoproteins *in vivo*.

Incubations of freshly isolated liver cells with the indicated amounts of lipoprotein and cells were performed in Ham's F-10 medium (modified), containing 2 % (v/v) BSA. The incubations were carried out either in plastic Eppendorf tubes in 0.5 ml total volume or in Kartell plastic tubes. A circulating laboratory shaker (Adolf Kühner AG, Switzerland) at 150 rpm was used. Viability as checked by Trypan Blue exclusion was above 95 % for all liver cell types used throughout the incubations. At the indicated times, 0.5 ml samples were withdrawn and the cells were centrifuged in an Eppendorf centrifuge for 2 min at 3000 rpm (735 ***g***). The pellets were suspended in 0.5 ml of medium containing 50 mM Tris/HCl, pH 7.4, 0.15 M NaCl, 2.5 mM $CaCl_2$ and 0.2 % BSA, incubated for 5 min at 4 °C, and centrifuged again. This washing procedure was repeated twice. The last washing was performed in medium without BSA to enable a reliable protein determination. The cell-associated radioactivity and 0.5 ml of the different supernatants were counted in a Packard gamma counter. The radioactivity in the last supernatant was < 5 % of the cell-associated radioactivity. Degradation of the lipoproteins was measured according to [36], as described in [34]. To 0.5 ml of the first supernatant, 0.2 ml of 35 % trichloro-

acetic acid (TCA) was added followed by incubation for 15 min at 37 °C; subsequently the mixture was centrifuged for 2 min at 15000 rpm (16000 ***g***). To 0.5 ml of the supernatants, 10 μl of 20 % KI and 25 μl of 30 % H_2O_2 were added. After 5 min at room temperature, 0.8 ml of $CHCl_3$ was added and the mixture was shaken for another 5 min. After centrifugation for 2 min at 15000 rpm (16000 ***g***), 0.4 ml of the aqueous phase (containing iodinated amino acids and small peptides) and 0.5 ml of the $CHCl_3$ phase (containing I_2 formed from I^- by oxidation with H_2O_2) were counted. This sample was corrected for quenching by $CHCl_3$. In the corresponding blanks the lipoproteins were incubated in the absence of cells. The degradation values given in the Figures represent counts of the aqueous phase. Further additions are indicated in the Figure legends.

RESULTS

Serum decay and liver association of Ac-LDL

The disappearance of Ac-LDL (5 μg) from the blood circulation in control mice proceeds at a rapid rate, and 2 min after injection more that 90 % is already removed (Figure 1). The liver is mainly responsible for this rapid removal and 5 min after injection 79.1 ± 4.6 % (± S.D., $n = 7$) of the injected dose is liver-associated. Subsequently the radioactivity within the liver declines.

Surprisingly, we observe no apparent difference in the clearance rate of Ac-LDL when injected into scavenger-receptor class A types I and II knock-out mice. Also the initial kinetics of liver uptake are similar to control mice [it was 77.4 ± 6.3 % ($n = 5$) 5 min after injection] whereas there was also no significant difference in the decline in liver-associated radioactivity.

It might be assumed that at a low dose of Ac-LDL (5 μg), the capacity of the uptake system cannot be assessed, thus we also injected a higher dose (200 μg, Figure 1). It appears that indeed the initial clearance is retarded but again no significant difference in clearance and liver uptake between control and scavenger-receptor knock-out mice is noticed indicating that scavenger-receptors distinct from class A types I and II can mediate efficient clearance of Ac-LDL from the blood circulation. Initial data on the clearance of Ac-LDL in knock-out mice and wild-type mice have already been reported [25].

To analyse whether the decline in liver-associated radioactivity of Ac-LDL starting 5 min after injection is caused by rapid degradation, we labelled Ac-LDL with ^{125}I-tyramine cellobiose and determined the conversion rate from TCA-precipitable to

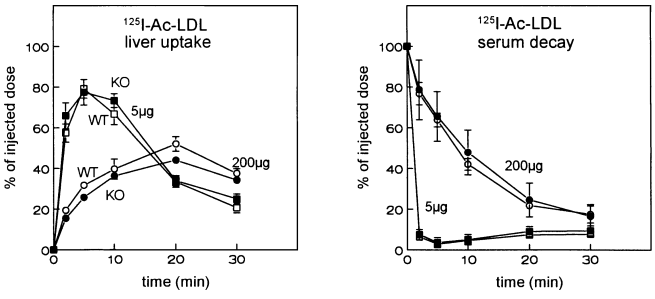

Figure 1 Liver uptake and serum decay of Ac-LDL at two doses in wild-type (WT) and knock-out (KO) mice

^{125}I-Ac-LDL (5 or 200 μg of apolipoprotein) was injected into anaesthetized WT (□, ○) and KO (■, ●) mice, and the liver association and serum decay was determined. Bars represent S.E. for 5–7 animals for 5 μg and 3 animals for 200 μg. The liver value is corrected for serum radioactivity based upon ^{3}H-labelled albumin.

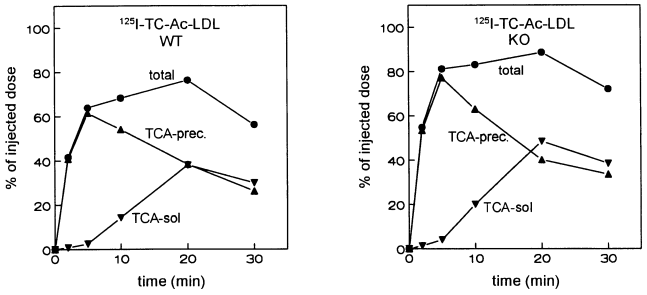

Figure 2 Liver uptake and degradation of ^{125}I-tyramine-cellobiose (TC) labelled Ac-LDL

^{125}I-TC-Ac-LDL (15 μg of apolipoprotein) was injected into anaesthetized wild-type (WT) and knock-out (KO) mice, and the liver association and conversion from TCA-precipitable to TCA-soluble products was determined at various times after injection. Data from one of two representative experiments is given.

TCA-soluble radioactivity within the liver in wild-type and knock-out mice (Figure 2). It appears that the total radioactivity remains associated with the liver for a prolonged time. Furthermore the appearance of TCA-soluble products starts between 5 and 10 min after injection and these increase linearly up to 20 min. No apparent difference between wild-type and knock-out mice was noticed. The data indicate that in wild-type and knock-out mice Ac-LDL is degraded at an equally rapid rate.

As an independent control, the decay rate and liver-uptake kinetics of radiolabelled asialo-orosomucoid (ASOR) was determined in wild-type and knock-out mice. ASOR is a specific substrate for the asialoglycoprotein receptor that resides specifically in the parenchymal liver cells [34]. The serum decay of ^{125}I-ASOR both in wild-type and scavenger-receptor class A types I and II knock-out mice is very fast and at 2 and 5 min after injection for wild-type mice only 7.0 ± 1.4 (± S.E., $n = 3$) and 1.3 ± 0.4 % (± S.E., $n = 3$) is still present in serum, while for knock-out mice these values were 6.3 ± 0.6 % and 1.4 ± 0.4 %, respectively. This fast decay was caused by a rapid liver uptake of ^{125}I-ASOR and 5 min after injection 98.1 ± 6.7 % (± S.E., $n = 3$) and 96.4 ± 4.9 % (± S.E., $n = 3$) of the injected dose was recovered in liver for wild-type and knock-out mice, respectively. These values clearly indicate that a ligand irrelevant to the scavenger-receptor is cleared from serum and taken up by the liver at a similar rate.

Cellular distribution of Ac-LDL in liver

We have shown previously in rats that Ac-LDL is mainly taken up by endothelial cells. In agreement with these data we find now, also for control mice, that the highest specific uptake (per mg of cell protein) is found to be associated with endothelial cells (Table 1). For the knock-out mice, essentially similar cellular uptake values were obtained, although the value for endothelial cells was 17 % lower ($p = 0.04$). When the amount of protein contributed to total liver by each cell type was taken into account, the contribution of each of the various liver cell types to total liver uptake could be calculated (Table 1). The mice endothelial cells indeed appear to be the main site for uptake of Ac-LDL. In principle, the relative contribution of the various cell types to the uptake of Ac-LDL appears to be similar for the knock-out and control mice.

Table 1 Distribution *in vivo* of ^{125}I-tyramine-cellobiose labelled Ac-LDL between parenchymal, endothelial and Kupffer cells of control and knock-out mice

At 10 min after injection of ^{125}I-tyramine-cellobiose labelled Ac-LDL (40 μg) the parenchymal, endothelial and Kupffer cells were isolated. The amount of radioactivity as percentage of the injected dose (ID) per mg of cell protein was determined and the relative contribution of the different liver cell types to the total uptake by liver is indicated, based upon the amount of protein that each cell type contributes to total liver protein [23].

	% ID/mg of cell protein		Relative contribution (%)	
Cell type	Control mice	Knock-out mice	Control mice	Knock-out mice
Parenchymal cells	0.01 ± 0.00	0.02 ± 0.00	2.8 ± 1.8	5.7 ± 0.2
Endothelial cells	7.24 ± 0.11	6.05 ± 0.39 ($p < 0.05$)	74.4 ± 1.2	71.8 ± 1.1
Kupffer cells	2.93 ± 0.07	2.53 ± 0.34	22.9 ± 0.6	22.6 ± 1.3

Table 2 Tissue distribution of Ac-LDL in wild-type and scavenger-receptor class A types I and II knock-out mice

^{125}I-Ac-LDL was injected intravenously into mice at a dose of 10 μg apolipoprotein. At 5 min after injection the indicated tissues were removed, weighed and counted for radioactivity. All values have been corrected for the presence of serum in the tissues. The values are means ± S.E. for 3 animals and only tissue values are given that contribute more than 2% of the injected dose. Total recovery of radioacitivity was 93–99%.

	% Injected dose	
	Control mice	Knock-out mice
Liver	66.6 ± 5.2	73.2 ± 3.4
Kidneys	3.5 ± 0.8	2.4 ± 0.9
Spleen	2.2 ± 0.1	3.2 ± 1.2
Skin	5.7 ± 1.0	4.8 ± 0.1
Muscles	4.4 ± 0.2	3.4 ± 0.5
Bones	22.3 ± 4.7	21.9 ± 3.2

Tissue distribution of Ac-LDL

In addition to the liver, scavenger-receptor-mediated uptake of Ac-LDL has been described for spleen, bone marrow and adrenal glands [37]. We analysed the tissue distribution of Ac-LDL 5 min after injection in knock-out and control mice (Table 2). It appears that indeed the liver is the major tissue site for the uptake of Ac-LDL with additional uptake by a variety of tissues, with bones that include bone-marrow as the second major site. No significant difference in tissue distribution of Ac-LDL between knock-out mice and control mice was observed (Table 2).

Effect of poly I *in vivo*

The binding of Ac-LDL to peritoneal macrophages can be inhibited by a wide variety of compounds including polyribonucleotides such as poly I [38]. The association of Ac-LDL with rat liver *in vivo* can also be greatly blocked by preinjection of poly I [23]. This poly I sensitivity is well described for scavenger-receptor class A types I and II and we rationalized that alternative scavenger-receptors, which are apparently involved in the uptake of Ac-LDL in the knock-out mice, might show poly I insensitivity. We therefore performed decay and liver-uptake studies of Ac-LDL after preinjection of poly I (Figure 3). It appears that both in the control and knock-out mice the liver uptake of Ac-LDL can be nearly completely blocked by a high concentration of poly I (Figure 3). This is also reflected in the serum decay of Ac-LDL,

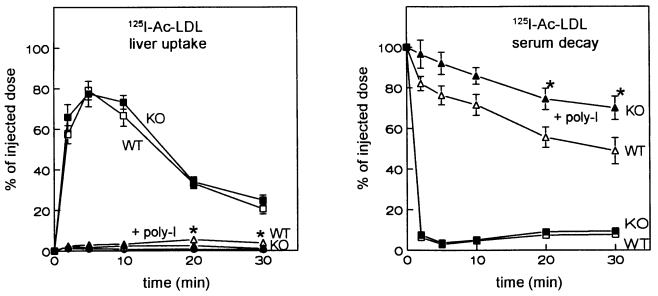

Figure 3 Effect of poly I on the liver uptake and serum decay of Ac-LDL

^{125}I-Ac-LDL (5 μg apolipoprotein) was injected into anaesthetized wild-type (WT) and knock-out (KO) mice. 1 min prior to injection of Ac-LDL, either solvent (■,□) or 200 μg of poly I were preinjected (▲,△). Open symbols represent WT and closed symbols KO mice. Bars represent S.E. for 5–7 animals. *, Significant difference ($p < 0.05$) by Student's t test (two sided). The serum decay in the presence of poly I between KO and WT mice differed highly significantly ($p < 0.0001$, analysis of variance).

leading to a strongly prolonged circulation time. The effect of 200 μg of poly I on the serum decay and liver uptake in knock-out versus wild-type mice suggested to us that poly I might be more effective in the knock-out mice (significant at 20 and 30 min after injection). Therefore we studied the serum decay and liver uptake of Ac-LDL also after preinjection of 20 and 50 μg of poly I (Figure 4). Interestingly, at suboptimal poly I concentrations we observe that poly I is more effective in blocking the decay of Ac-LDL from the blood of knock-out mice (Figure 4), which is

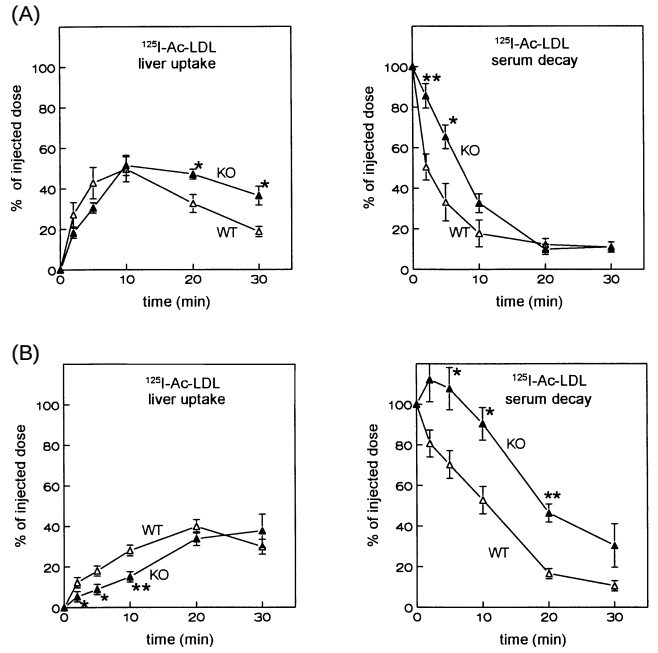

Figure 4 Effect of two concentrations of poly I on the liver uptake and serum decay of Ac-LDL in wild-type (WT) and knock-out (KO) mice

^{125}I-Ac-LDL (5 μg apolipoprotein) was injected into anaestethized WT and KO mice. 1 min prior to injection of Ac-LDL either 20 μg (A) or 50 μg (B) of poly I was preinjected (▲,△). Open symbols represent WT and closed symbols KO mice. Bars represent S.E. for 3 animals. *, Significant difference ($p < 0.05$); **, very significant difference ($p < 0.01$) by Student's t test (two sided). The serum decay in the presence of poly I between KO mice and WT mice differed highly significantly ($p < 0.0001$, analysis of variance).

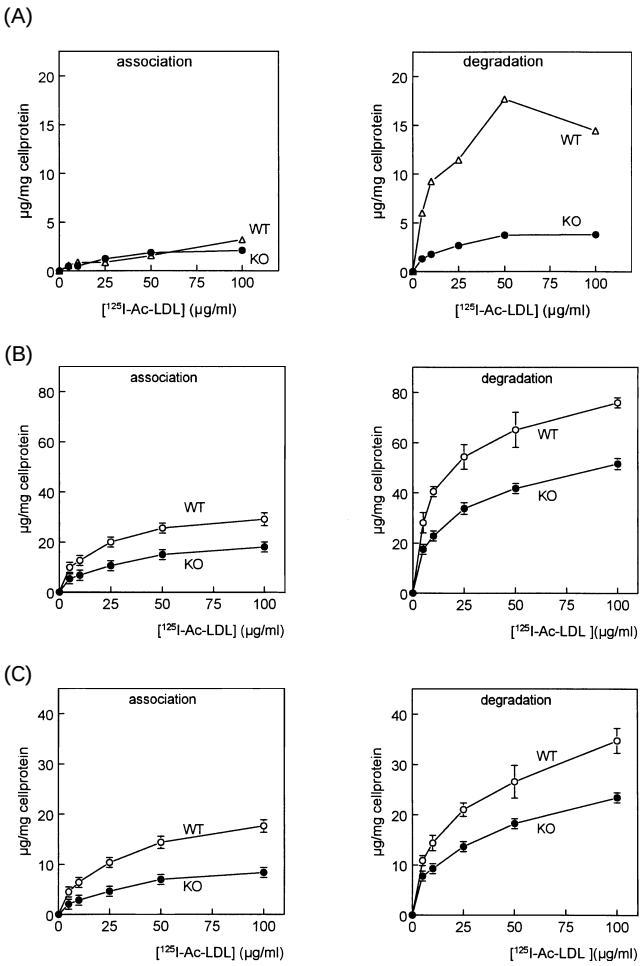

Figure 5 Cell association and degradation of Ac-LDL by isolated peritoneal macrophages (A), endothelial (B) or Kupffer cells (C) from wild type (WT) and knock-out (KO) mice as function of the Ac-LDL concentration

The amount of ^{125}I-Ac-LDL was varied and the cell association or degradation is expressed as mg of apolipoprotein/mg of cell protein. Incubation time was 2 h at 37 °C and the values represent the mean ± S.E. ($n = 3$) for endothelial and Kupffer cells while peritoneal macrophages were isolated from 30 WT or 30 KO mice. Open symbols represent WT and closed symbols knock-out mice.

caused by a more effective inhibition of the liver uptake; this is especially significant at 50 μg of poly I. These data form the first evidence that a difference in Ac-LDL decay and liver uptake *in vivo* can indeed be noticed in the knock-out versus the wild-type mice, albeit in the presence of poly I.

Cell association and degradation of Ac-LDL by isolated cells

It was recently established that the degradation of Ac-LDL in thioglycolate-elicited peritoneal macrophages is reduced to less than one third in knock-out mice versus control mice [25]. In Figure 5(A) it is indicated that the cell association of Ac-LDL with peritoneal macrophages of knock-out mice and wild-type mice is comparable, whereas the degradation in knock-out mice reached 17 % of that in cells from wild-type mice, which is in agreement with the earlier data. Similar experiments were performed with endothelial and Kupffer cells isolated from mouse liver (Figures 5B and 5C). The cell association and degradation of Ac-LDL by endothelial cells follows saturation kinetics while for the cells isolated from knock-out mice the maximal cell association reaches a value which is 50 % of the control cells. For Ac-LDL degradation with endothelial cells from the knock-out mice the maximal value of degradation is 58 % of that found with the wild-type endothelial cells (Table 3).

For isolated Kupffer cells it also appears that the cell association and degradation of Ac-LDL is lower for the cells from the scavenger-receptor class A types I and II knock-out mice and the maximal cell association with the cells from knock-out mice reaches 53 % of the control while for the degradation a value of 63 % is observed. It thus appears that for the isolated cells a significant proportion of the uptake and degradation is exerted by scavenger-receptor class A types I and II. In agreement with data obtained from rat liver cells [22], the metabolism of Ac-LDL by endothelial liver cells from control mice is more active (per mg of cell protein) than observed with Kupffer cells.

Cell binding of Ac-LDL by isolated cells

It can be argued that the differences in degradation of Ac-LDL by cells from knock-out mice versus wild-type mice might be caused by a lower rate of receptor ligand internalization or by a lower number of surface receptors. To discriminate between these two possibilities we performed binding studies at 4 °C (Figure 6). It appears that indeed the binding of Ac-LDL to the endothelial cells and Kupffer cells from the knock-out mice is about half of that of cells from the wild-type mice, indicating a decrease in surface receptors in the cells from knock-out mice. It can also be noticed that the difference in binding between cells from knock-out mice versus wild-type mice is more evident at higher Ac-LDL concentrations (above 25 μg/ml), suggesting that the remaining scavenger-receptors in knock-out mice possess a relatively high affinity for Ac-LDL.

Table 3 Capacities of cell association and degradation of Ac-LDL by Kupffer cells and endothelial liver cells from wild-type and scavenger-receptor class A types I and II knock-out mice

Substrate curves ($n = 3$) were analysed according to a single-site binding model using non-linear regression (Graph-PAD, ISIS Software).

	Kupffer cells [Apparent V_{max} (μg/mg of cell protein)]		Endothelial cells [Apparent V_{max} (μg/mg of cell protein)]	
	Cell association	Degradation	Cell association	Degradation
Wild-type mice	15.1 ± 2.2	21.4 ± 1.1	28.3 ± 4.2	65.0 ± 1.3
Knock-out mice	8.0 ± 2.5	13.5 ± 1.7	14.1 ± 3.1	37.8 ± 2.7
Relative percentage of knock-out versus wild-type mice	53.0%	63.0%	49.8%	58.2%

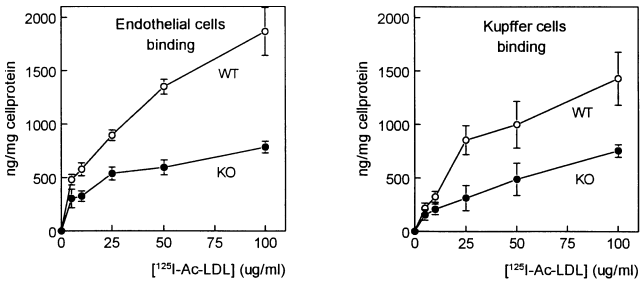

Figure 6 Cell binding of Ac-LDL by isolated endothelial or Kupffer cells from wild type (WT) and knock-out (KO) mice as function of the Ac-LDL concentration

The amount of ^{125}I-Ac-LDL was varied and the cell binding is expressed as ng of apolipoprotein/mg of cell protein. Incubation time was 2 h at 4 °C and the values represent the mean ± S.E. ($n = 3$). Open symbols represent WT and closed symbols knock-out mice.

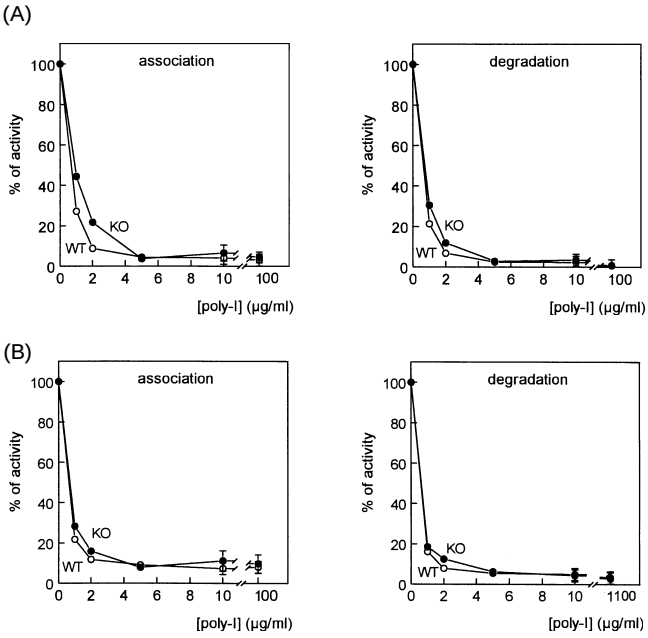

Figure 7 Effect of poly I on the cell association and degradation of Ac-LDL by isolated endothelial (A) or Kupffer cells (B) from wild type (WT) and knock-out (KO) mice

The amount of poly I was varied and values represent the mean ± S.E. ($n = 3$), when indicated by a bar. The concentration of ^{125}I-Ac-LDL was 5 μg/ml and the incubation time was 2 h at 37 °C. Open symbols represent WT and closed symbols KO mice.

Effect of poly I *in vitro*

The more effective inhibition of poly I on the Ac-LDL clearance and liver uptake in the knock-out mice versus the wild-type mice might be caused by a higher sensitivity of the alternative scavenger-receptors in the knock-out mice for poly I, or to a decrease in binding sites. To discriminate between these two possibilities we determined with isolated endothelial and Kupffer cells the effect of varying poly I concentrations on the cell association and degradation of Ac-LDL (Figure 7). It appears that poly I is a very effective inhibitor for the cell association and degradation of Ac-LDL both in endothelial and Kupffer cells from knock-out mice and control mice. Poly I appears not to be more effective in knock-out mice versus control mice, so we can conclude that the significant differences *in vivo* must be caused by the different number of binding sites.

DISCUSSION

Scavenger-receptors are implicated in the pathological deposition of cholesterol (esters) during atherogenesis. The cloning and characterization [11,12] of scavenger-receptor class A types I and II has now led to the creation of receptor-deficient mice [25]. In addition to Southern-blot analysis, receptor deficiency has also been shown by the absence of immunostaining by the anti-murine scavenger-receptor class A types I and II monoclonal antibody (2F8) of liver sinusoidal cells in the knock-out mice [25]. More than a decade ago we showed in rats that upon injection of Ac-LDL, these particles were rapidly removed by the blood circulation and that liver endothelial cells were the main site of uptake [22]. We assumed that the scavenger-receptor class A types I and II were responsible for this uptake because the interaction of Ac-LDL with liver endothelial and Kupffer cells *in vitro* possessed characteristics similar to those described for this type of scavenger-receptor [38]. It is therefore surprising to observe that upon injection of Ac-LDL into knock-out mice a rapid clearance and liver uptake is observed. Moreover, the mouse liver endothelial cell also appears to be the major liver site for Ac-LDL uptake both in control and receptor-deficient mice. We do not consider the 17 % inhibition in uptake of Ac-LDL by endothelial cells in the knock-out mice to be a very significant reflection of receptor deficiency (although $p < 0.05$). Also the injection of a higher dose of Ac-LDL (200 μg) did not lead to a significant effect on the decay or liver uptake, although the kinetics clearly point to a partial saturation of the uptake system. From these data, we have to conclude that alternative uptake systems for Ac-LDL have to be present in the liver endothelial and Kupffer cells, which can compensate for the absence of scavenger-receptor class A types I and II, and that therefore this receptor concentration is not rate limiting *in vivo*. Furthermore, the degradation of Ac-LDL was not hampered *in vivo* in the knock-out mice.

One of the characteristic properties of scavenger-receptor class A types I and II is their sensitivity for poly I. Previously we have shown in rats that preinjection of poly I *in vivo*, before Ac-LDL administration, greatly blocks the liver uptake [23]. To verify whether the alternative uptake system for Ac-LDL in the knock-out mice is sensitive to poly I, we preinjected various concentrations. It is clear that also in the knock-out mice poly I is an effective inhibitor for the liver uptake of Ac-LDL, as also reflected in the prolonged circulation time. At suboptimal poly I concentrations, it appears that the liver uptake and serum decay of Ac-LDL is more effectively inhibited by poly I in the knock-out mice (especially evident at 50 μg of poly I). These data might indicate that knock-out mice do possess fewer binding sites for poly I, leading to a higher effective concentration for inhibition of the alternative scavenger-receptors, or that the alternative scavenger-receptors are more sensitive to poly I. To discriminate between these two possibilities, the sensitivity of cell association and degradation of Ac-LDL for poly I was determined *in vitro*. It appears that both in cells from knock-out mice and wild-type mice a similar effectivity for inhibition can be observed, suggesting that the more effective inhibition of Ac-LDL clearance and liver uptake by poly I in the knock-out mice *in vivo* is caused by a decrease in binding sites for poly I.

Studies i*n vitro* with isolated mouse endothelial, Kupffer and peritoneal macrophages were also performed to assess whether the capacity of the individual cell types to interact with Ac-LDL

is hampered by the absence of scavenger-receptor class A types I and II. For peritoneal macrophages it is clear, as also briefly reported recently [25], that the degradation of Ac-LDL is affected (in knock-out cells the maximal degradation is 17% of the cells from control mice). Lougheed et al. [39] also recently reported an 80% reduction of Ac-LDL degradation in macrophages from scavenger-receptor class A types I and II knock-out mice. For the isolated endothelial and Kupffer cells we observe that the maximal degradation of Ac-LDL reaches in the cells from knock-out mice values of 58 and 63%, respectively, of the cells from wild-type mice. These data indicate that the scavenger-receptor class A types I and II is of quantitative importance for the degradation of Ac-LDL by these cell types. Cell-binding experiments for Ac-LDL, performed at 4 °C, indicate that the lower capacity for Ac-LDL degradation in knock-out mice versus wild-type mice is caused by a decrease in surface receptors on the cells. The surprising observation that a 40–50% reduction in cell binding, cell association and degradation of Ac-LDL by knock-out mice liver endothelial and Kupffer cells *in vitro* is not reflected in a concomitant delay in clearance *in vivo* might be caused by the extremely rapid recycling of the alternative scavenger-receptors. It might also be possible that initial binding *in vivo* to proteoglycans may mask the receptor deficiency. Apparently an excess capacity is present *in vivo* for the turnover of modified lipoproteins and other potential ligands for scavenger-receptors. It must be concluded that alternative, still-unidentified receptors are present which can take over the function of scavenger-receptor class A types I and II. Whether these receptors are related to recently described scavenger-receptors such as Marco [17], macrosialin [18,19], CD 36 [20] and scavenger-receptor B1 [21] is presently unknown and awaits characterization. However, some of these candidate proteins like CD 36 and scavenger-receptor class B1 can already be excluded, because the effective inhibition of the liver uptake of Ac-LDL in the knock-out mice by poly I indicates that these alternative systems are highly sensitive to poly I. Furthermore macrosialin is specifically localized in Kupffer cells [19], ruling out a function for this protein in liver endothelial cells. Clearly the availability of the scavenger-receptor class A types I and II knock-out mice will enable the functional evaluation of up-until-now molecularly unidentified scavenger-receptor-like uptake systems.

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