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Delivery of cholesteryl-conjugated phosphorothioate oligodeoxynucleotides to Kupffer cells by lactosylated low-density lipoprotein

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Abstract

The efficacy of antisense oligonucleotides depends on the ability to reach *in vivo* their target cells. We aim to develop strategies to enhance uptake of phosphorothioate oligodeoxynucleotides by Kupffer cells. To this end, we conjugated cholesterol to ISIS-3082, a phosphorothioate oligodeoxynucleotide specific for intercellular adhesion molecule-1. The cholesterol-conjugated oligonucleotide, denoted ISIS-9388, associated readily with lactosylated low-density lipoprotein (LacLDL), a lipidic carrier that is taken up by galactose receptors on Kupffer cells. Association of up to 10 molecules of ISIS-9388 per LacLDL particle did not induce aggregation. LacLDL-associated [^{3}H]ISIS-9388 was rapidly taken up by the liver after injection into rats (52.9 ± 1.8% of the dose within 2 min versus 18.6 ± 2.8% for ISIS-3082). *N*-acetylgalactosamine inhibited hepatic uptake, indicating involvement of galactose-specific receptors. Liver cells were isolated at 60 min after injection of LacLDL-associated [^{3}H]ISIS-9388. Kupffer cells displayed the highest uptake: 88.1 ± 24.7 ng of oligonucleotide/mg of cell protein, which is 6–14 times higher than after injection of free ISIS-9388 or ISIS-3082 (15.0 ± 3.8 ng and 6.3 ± 1.4 ng, respectively). It can be calculated that Kupffer cells contribute 43.9 ± 5.4% to the liver uptake (free ISIS-9388 and ISIS-3083 14.5 ± 3.1% and 8.3 ± 3.2%, respectively). In conclusion, conjugation of a phosphorothioate oligodeoxynucleotide with cholesterol and its subsequent association with LacLDL results in a substantially increased Kupffer cell uptake of the oligonucleotide. As Kupffer cells play a key role in inflammation, our approach may be utilized to improve antisense-based therapeutic intervention during inflammation.

Keywords: Antisense oligodeoxynucleotides; Cholesterol conjugation; ICAM-1; Kupffer cells; Lactosylated low-density lipoprotein; Liver

1. Introduction

The ability of antisense oligonucleotides to interdict sequence-specifically the expression of pathogenic genes affords an exiting new strategy for therapeutic intervention [1–3]. Numerous oligonucleotide analogues have been synthesized in order to obtain compounds with optimal pharmacokinetic and pharmacodynamic characteristics. Oligodeoxynucleotides containing the phosphorothioate backbone are the most extensively studied. Phosphorothioate oligodeoxynucleotides have been shown to be potent inhibitors of gene expression, both *in vitro* and *in vivo* [1–3]. Several are in clinical trial and one, Vitravene™, has been approved for marketing.

The therapeutic efficacy of antisense oligonucleotides depends largely on the capacity to reach their target cells *in vivo* in sufficient quantities. Preclinical studies indicate that the liver is the main site of disposition of phosphorothioate oligodeoxynucleotides [3]. We showed recently that within the liver uptake occurs predominantly by endothelial cells, whereas Kupffer accumulate much less oligonucleotide [4]. However, Kupffer cells constitute an important target for therapeutic intervention. These cells play a key role during inflammation. The expression of adhesion molecules on Kupffer cells is enhanced under inflammatory conditions, which results in the harmful infiltration of neutrophils into

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Abbreviations: ICAM-1, intercellular adhesion molecule-1; LDL, low-density lipoprotein; and LacLDL, lactosylated low-density lipoprotein.

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the liver [5,6]. Furthermore, Kupffer cells produce during inflammation mediators such as tumor necrosis factor-alpha, interleukins, and prostanoids that start a cascade of events leading to serious disease [6].

Our aim is to enhance the uptake of phosphorothioate oligodeoxynucleotides by Kupffer cells. Our approach is to associate the oligonucleotides with carriers that are specifically taken up by Kupffer cells. In the present study, we used lactosylated low-density lipoprotein (LDL) as carrier. Lactosylated LDL consists of a lipid moiety and an apoprotein that is derivatized with galactose residues [7,8]. The exposed galactose residues induce rapid and very selective uptake of the particles by Kupffer cells [7]. Uptake of lactosylated LDL by Kupffer is mediated by galactose-particle receptors. These receptors, which are different from the classical asialoglycoprotein receptor [9], are only expressed on Kupffer cells [7]. The lipid moiety of lactosylated LDL can be utilized to associate a variety of lipophilic (pro)drugs with the particle.

In the present study, we associated ISIS-3082, a phosphorothioate oligodeoxynucleotide specific for the murine intercellular adhesion molecule-1 (ICAM-1), with lactosylated LDL. To enable incorporation into the lipid moiety of lactosylated LDL, ISIS-3082 was conjugated at its 3′ end with cholesterol. The cholesterol-derivatized ISIS-3082 was denoted ISIS-9388. Delivery of the lactosylated LDL-associated ISIS-9388 to Kupffer cells is expected to down-regulate ICAM-1 expression on these cells, which will reduce or prevent the harmful infiltration of neutrophils. We investigated the interaction of ISIS-9388 with lactosylated LDL, and examined the disposition and liver cell uptake of complexes of ISIS-9388 and lactosylated LDL.

2. Materials and methods

2.1. Materials

Emulsifier Safe™ and Hionic Fluor™ scintillation cocktails and Soluene-350 were from Packard. $Na^{125}I$ (carrier-free) was from Amersham International. All other reagents were of analytical grade.

2.2. Oligonucleotide synthesis and radiolabeling

ISIS-3082, a 20-mer phosphorothioate oligodeoxynucleotide specific for murine ICAM-1 (sequence 5′-TGC ATC CCC CAG GCC ACC AT-3′), was synthesized as described before [10]. ISIS-9388 has the same sequence as ISIS-3082, except that the 3′-end base thymidine is replaced by a modified uridine, to which cholesterol is attached via a linker. ISIS-9388 was synthesized as described earlier [11]. To allow monitoring the biological fate of both oligonucleotides, they were radiolabeled with ^3H by heat-catalyzed exchange at the C8 positions of the purine nucleotides as described previously [12]. The specific radioactivities of [^{3}H]ISIS-3082 and [^{3}H]ISIS-9388 were approximately 50×10^6 dpm/mg, and the radiochemical purity >90% [4,11].

2.3. Preparation of (radioiodinated) lactosylated LDL

Human low-density lipoprotein (density 1.019–1.063 g/mL) was isolated by two consecutive density gradient centrifugation steps, as described earlier [13]. Lactosylated LDL was prepared by reductive lactosamination, and radiolabeled with ^{125}I, as described in detail earlier [7].

2.4. Determination of plasma clearance and tissue distribution

Male Wistar rats, weighing between 200 and 350 gram, were used. The animals were anaesthesized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight), and the abdomen was opened. Radiolabeled oligonucleotides (free or associated with lactosylated LDL), dissolved in phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl), were injected via the vena penis (2 mL/kg body weight). At the indicated times, blood samples of 0.2–0.3 mL were taken from the inferior vena cava and collected in heparinized tubes. The samples were centrifuged for 2 min at $16{,}000 \times g$, and the plasma was assayed for radioactivity. The total amount of radioactivity in plasma was calculated using the equation: plasma volume (mL) = [0.0219 × body weight (g)] + 2.66 [7]. At the indicated times, liver lobules were tied off and excised, and at the end of the experiment the remainder of the liver was removed. The amount of liver tissue tied off successively did not exceed 15% of the total liver mass. The amount of radioactivity in the liver at each time point was calculated from the radioactivities and weights of the liver samples. Uptake by extrahepatic tissues was determined by removing the tissues at the end of the experiment, and counting of radioactivity. Radioactivity in the tissues was corrected for radioactivity in plasma present in the tissue at the time of sampling [14].

2.5. Determination of the distribution over liver cell types

Rats were anaesthesized and injected with radiolabeled oligonucleotides as described above. Sixty minutes later, parenchymal, Kupffer, and endothelial cells were isolated from the liver as described in detail earlier [15]. The cell fractions were assayed for radioactivity and protein. The contributions of the various cell types to the total liver uptake was calculated as described previously [15]. As found with other ligands [7,15], no significant amounts of radioactivity were lost from the cells during isolation. This was checked in each experiment by comparing the calculated liver uptake (i.e. the summation of the contributions of the various cell types) with the value actually measured in the liver lobule.

2.6. Analysis of association of oligonucleotides with lactosylated LDL: size exclusion chromatography

[^{125}I]lactosylated LDL (200 μg/mL) was incubated at 37° with [^{3}H]ISIS-9388 (2.5–25 μg/mL) or [^{3}H]ISIS-3082 (25 μg/mL). After 30 min, 50 μL aliquots of the incubation mixtures were injected onto a Superose 6 Precision Column (3.2 mm × 300 mm; SMART system, Pharmacia). The column was eluted with phosphate-buffered saline at a flow rate of 50 μL/min. Fractions of 100 μL were collected and assayed for radioactivity.

2.7. Analysis of association of oligonucleotides with lactosylated LDL: agarose gel electrophoresis

[^{125}I]lactosylated LDL (200 μg/mL) was incubated at 37° with [^{3}H]ISIS-9388 (2.5–25 μg/mL) or [^{3}H]ISIS-3082 (25 μg/mL). After 30 min, aliquots of the incubation mixtures were subjected to electrophoresis in a 0.75% (w/v) agarose gel at pH 8.8 (75 mM Tris-hippuric acid buffer). Bromophenol blue was used as front marker. After electrophoresis, the gel was sliced and the slices were assayed for radioactivity.

2.8. Determination of proteins

Protein concentrations in cell suspensions and lactosylated LDL preparations were determined by the method of Lowry *et al*. [16], with a bovine serum albumin standard.

2.9. Determination of radioactivity

Samples containing ^{3}H were counted in a Packard Tri-Carb 1500 liquid scintillation counter. Liquid samples were counted without further processing, using Emulsifier Safe™ or Hionic Fluor™ scintillation cocktails. Agarose gel slices were first dissolved in *N*-methyl-2-pyrrolidone. Tissue samples were processed using a Packard 306 Sample Oxidizer. Samples containing both ^{125}I and ^{3}H, were first assayed for ^{125}I radioactivity using a Packard Auto-Gamma 5000 counter. The ^{3}H radioactivity was subsequently measured as described above and corrected for the contribution of ^{125}I radioactivity.

3. Results

3.1. Preparation and characterization of complexes of lactosylated LDL with ISIS-9388

To enable the association of ISIS-3082 with lactosylated LDL, we synthesized ISIS-9388, the 3′-cholesteryl-conjugated derivative of ISIS-3082. The association of ISIS-9388 with lactosylated LDL was examined by mixing the oligonucleotide and the carrier at ratios of 1, 5, or 10 oligonucleotide molecules per lactosylated LDL particle, and incubating for 30 min at 37°. Lactosylated LDL and ISIS-9388 were radiolabeled with ^{125}I and ^{3}H, respectively, to allow monitoring of both the carrier and the oligonucleotide. The incubation mixtures were analyzed by size exclusion chromatography. Fig. 1, A–C shows that at all ratios both radiolabels eluted at the position of the carrier. No significant amounts of ^{3}H radioactivity (i.e. ISIS-9388) were recovered at the position of free oligonucleotide. This finding indicates that all added oligonucleotide associates with lactosylated LDL, and that association of ISIS-9388 does not result in aggregation of the lipoprotein particle. Fig. 1D shows that the unconjugated oligonucleotide ISIS-3082 does not associate with lactosylated LDL, which indicates that the 3′-cholesterol moiety of ISIS-9388 is essential for its interaction with lactosylated LDL.

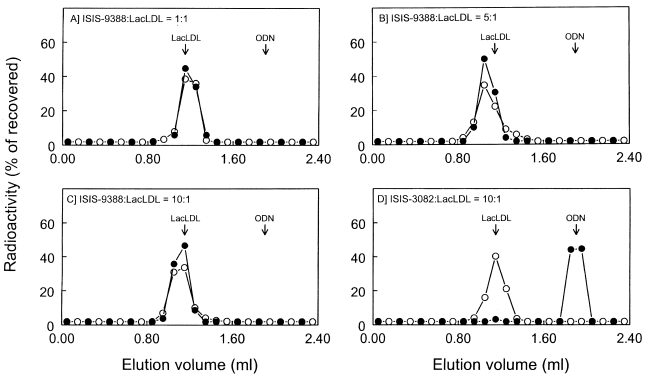

Fig. 1. Interaction of ISIS-9388 and ISIS-3082 with lactosylated LDL: analysis by size exclusion chromatography. [^{125}I]Lactosylated LDL (200 μg/mL) was incubated at 37° with [^{3}H]ISIS-9388 at concentrations of 2.5, 12.5, or 25.0 μg/mL (panels A–C; molar ratios ISIS-9388:lactosylated LDL of 1:1, 5:1, and 10:1, respectively), or with [^{3}H]ISIS-3082 at a concentration of 25.0 μg/mL (panel D; molar ratio ISIS-3082:lactosylated LDL = 10:1). After 30 min, aliquots of the incubation mixtures were subjected to size exclusion chromatography using a calibrated Superose 6 column. The fractions (0.1 mL) were assayed for ^{3}H radioactivity (●) and ^{125}I radioactivity (○). The results are expressed as percentages of the recovered amounts (recoveries >65%). The elution volumes of lactosylated‘ LDL (LacLDL) and free oligodeoxynucleotide (ODN) are indicated by arrows.

To investigate the effect of the presence of the negatively charged ISIS-9388 on the electric charge of the complexes of ISIS-9388 with lactosylated LDL, mixtures of lactosylated LDL and ISIS-9388 were subjected to agarose gel electrophoresis. Fig. 2A shows that the electrophoretic mobility of complexes with 1 oligonucleotide per lactosylated LDL particle was not appreciably different from that of the particle alone (Rf 0.26). However, complexes with 5 or 10 oligonucleotides per lactosylated LDL particle displayed a significantly increased electrophoretic mobility (Rf values 0.38 and 0.49, respectively; Fig. 2, B and C), and thus an increased net negative charge. Fig. 2D shows that ISIS-3082 does not associate with lactosylated LDL.

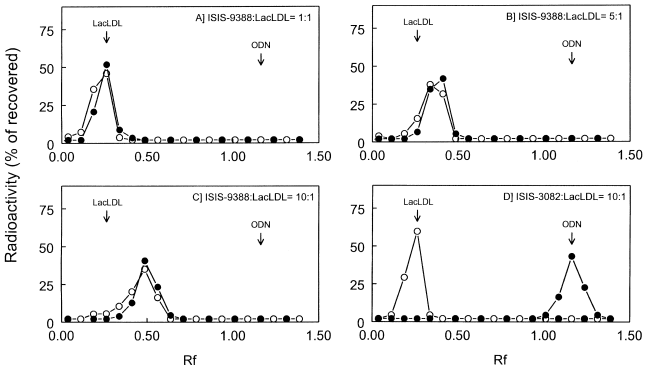

Fig. 2. Interaction of ISIS-9388 and ISIS-3082 with lactosylated LDL: analysis by agarose gel electrophoresis. [^{125}I]Lactosylated LDL (200 μg/mL) was incubated at 37° with [^{3}H]ISIS-9388 at concentrations of 2.5, 12.5, or 25.0 μg/mL (panels A–C; molar ratios ISIS-9388:lactosylated LDL of 1:1, 5:1, and 10:1, respectively), or with [^{3}H]ISIS-3082 at a concentration of 25.0 μg/mL (panel D; molar ratio ISIS-3082:lactosylated LDL = 10:1). After 30 min, aliquots of the incubation mixtures were subjected to electrophoresis in a 0.75% (w/v) agarose gel at pH 8.8 (75 mM Tris–hippuric acid buffer). Slices of the gel were assayed for ^{3}H radioactivity (●) and ^{125}I radioactivity (○). The amounts of radioactivity are given as percentage of the recovered amount (recoveries >80%). Migration (Rf) is given relative to the migration of Bromophenol blue (5.0 cm). The migrations of lactosylated LDL (LacLDL) and free oligodeoxynucleotide (ODN) are indicated by arrows.

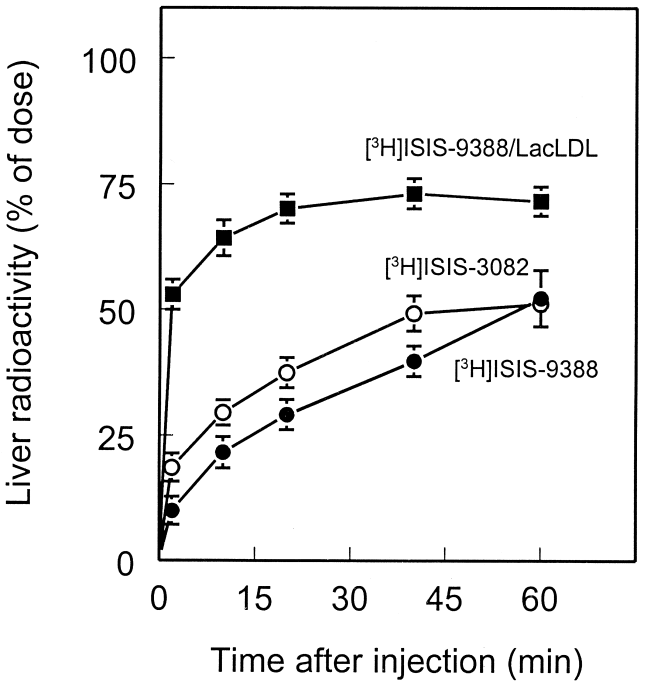

Fig. 3. Liver accumulation of lactosylated LDL-associated ISIS-9388, free ISIS-9388, and ISIS-3082. Rats were intravenously injected with lactosylated LDL-associated [^{3}H]ISIS-9388 (■), [^{3}H]ISIS-9388 (●), or [^{3}H]ISIS-3082 (○), all at a dose of 25 μg of oligonucleotide/kg body weight. At the indicated times, the amounts of radioactivity in the liver were determined. Values are means ± SEM of 3 rats.

3.2. Liver uptake of lactosylated LDL-associated ISIS-9388

To examine the effect of association of ISIS-9388 with lactosylated LDL on the liver uptake of the oligonucleotide, [^{3}H]ISIS-9388 was mixed with lactosylated LDL at a molar ratio of 5:1 (oligonucleotide:carrier), incubated for 30 min at 37°, and intravenously injected into rats. Fig. 3 shows that the carrier-associated oligonucleotide was very rapidly taken up by the liver. At 2 min after injection, the liver contained 52.9 ± 0.8% of the dose. The amount of liver-associated radioactivity subsequently gradually increased to 71.6 ± 2.9% of the dose at 60 min after injection. At that time, >90% of the radioactivity had been cleared from the plasma, and Table 1 shows that other organs such as muscles, spleen, and kidney contained only minor amounts of radioactivity. The highest extrahepatic uptake was by bone marrow. However, the amount recovered in the bone marrow (7.2 ± 0.4% of the dose) was still one order of magnitude lower than the amount recovered in the liver. Fig. 3 also shows the liver uptake of [^{3}H]ISIS-9388 when injected alone, and the liver uptake of the unconjugated parent compound [^{3}H]ISIS-3082. It is evident that the liver uptake of the carrier-associated ISIS-9388 proceeds much faster than that of both free oligonucleotides. The liver uptake of free ISIS-9388 proceeds more slowly than that of ISIS-3082, and reaches a maximum value of about 70% of the dose at 3 hr after injection (not shown). The liver accumulation of lactosylated LDL-associated ISIS-9388 and ISIS-3082 is maximal at 60 min after injection.

To ascertain that the rapid liver association of the complex of ISIS-9388 and lactosylated LDL is indeed mediated by galactose receptors, rats were preinjected with *N*-acetylgalactosamine prior to injection of the complexes of [^{3}H]ISIS-9388 with lactosylated LDL. Preinjection with *N*-acetylgalactosamine reduced the hepatic uptake of LacLDL-associated [^{3}H]ISIS-9388 by approximately 50%, whereas pretreatment with the same dose of *N*-acetylglucosamine had no significant effect (Table 2). This finding indicates that galactose-specific recognition sites in the liver are involved in the uptake.

Table 1
Tissue distribution of intravenously injected lactosylated LDL-associated ISIS-9388

Tissue	Radioactivity[a,b] (% of dose)
Blood plasma	9.2 ± 0.9
Liver	71.6 ± 2.9
Spleen	1.0 ± 0.1
Bone marrow	7.2 ± 0.4
Kidneys	1.8 ± 0.1
Intestines	1.5 ± 0.2
Muscles	1.3 ± 0.2
Skin	2.3 ± 0.5

[a] Rats were intravenously injected with lactosylated LDL-associated [^{3}H]ISIS-9388 (25 μg of oligonucleotide/kg body weight). At 60 min after injection, the distribution of radioactivity over the indicated tissues (which contained collectively >95% of the dose) was determined.

[b] Values are means ± SEM of 3 rats.

Table 2
Effects of *N*-acetylgalactosamine and *N*-acetylglucosamine on the liver uptake of lactosylated LDL-associated ISIS-9388

Preinjection[a]	Liver uptake (% of dose)[b,c,d]
Saline	59.0 ± 8.0
N-Acetylgalactosamine	26.7 ± 4.7*
N-Acetylglucosamine	48.6 ± 5.2[ns]
None	52.9 ± 0.8[ns]

[a] Rats were intravenously injected with lactosylated LDL-associated [^{3}H]ISIS-9388 (25 μg of oligonucleotide/kg body weight). One minute before injection, the animals received 400 mg of *N*-acetylgalactosamine/kg body weight, 400 mg of *N*-acetylglucosamine/kg body weight, or solvent (phosphate-buffered saline).

[b] At 2 min after injection of the radiolabeled complexes, the association of radioactivity to the liver was determined.

[c] Values are means ± SEM of 3–4 rats.

[d] Differences with respect to the saline controls were tested for significance by Wilcoxon's two-sample test [29].

* $P < 0.05$; ns = not significant.

3.3. Uptake of lactosylated LDL-associated ISIS-9388 by liver cell types

The liver contains several actively endocytosing cell types [9,17], and we demonstrated earlier that ISIS-3082 and ISIS-9388 are predominantly taken up by endothelial liver cells [4,11]. To examine the distribution of lactosylated LDL-associated ISIS-9388 over liver cell types, [^{3}H]ISIS-9388 was mixed with lactosylated LDL at a molar ratio of 5:1 (oligonucleotide:carrier), incubated for 30 min at 37°, and intravenously injected into rats. Parenchymal, endothelial, and Kupffer cells were isolated from the liver 60 min later. The cell isolation procedure was performed at a low temperature (8°) to prevent processing of internalized oligonucleotide. Fig. 4 shows the amounts of oligonucleotides present in the three isolated liver cell populations. The uptake of lactosylated LDL-associated ISIS-9388 by the liver cell types is compared with that of free ISIS-9388 and free ISIS-3082. As has been reported recently, all three liver cell types accumulate more of the cholesterol-modified ISIS-9388 than free ISIS-3082 [11]. However, if ISIS-9388 is associated with lactosylated LDL, a dramatic increase in uptake by Kupffer cells is observed. After injection of the complexes of ISIS-9388 with lactosylated LDL, Kupffer cells contained 88.1 ± 24.7 ng of oligonucleotide/mg of cell protein (Fig. 4), which is 6–14 times higher than after injection of free ISIS-9388 or ISIS-3082 (15.0 ± 3.8 and 6.3 ± 1.4 ng per mg of cell protein, respectively). In animals injected with lactosylated LDL-associated ISIS-9388, the level of oligonucleotide was highest in Kupffer cells. These cells contained 88.1 ± 24.7 ng of oligonucleotide/mg of cell protein, whereas endothelial and parenchymal cells contained 59.8 ± 17.4 and 0.8 ± 0.2 ng of oligonucleotide/mg of cell protein, respectively. From the uptake per mg of cell protein and the contribution of each cell type to the total liver protein, it can be calculated that Kupffer cells account for 43.9 ± 5.4% of the total liver uptake of the complexes if ISIS-9388 with lactosylated LDL (Table 3). Parenchymal liver cells (which display a low specific uptake but constitute >90% of the liver mass) were responsible for 16.7 ± 6.8% of the hepatic uptake, whereas endothelial cells accounted for 39.4 ± 3.8%. Thus, Kupffer cells contribute significantly to the liver uptake of lactosylated LDL-associated ISIS-9388, which is clearly different from the situation after injection of free ISIS-9388 and ISIS-3082 (Table 3).

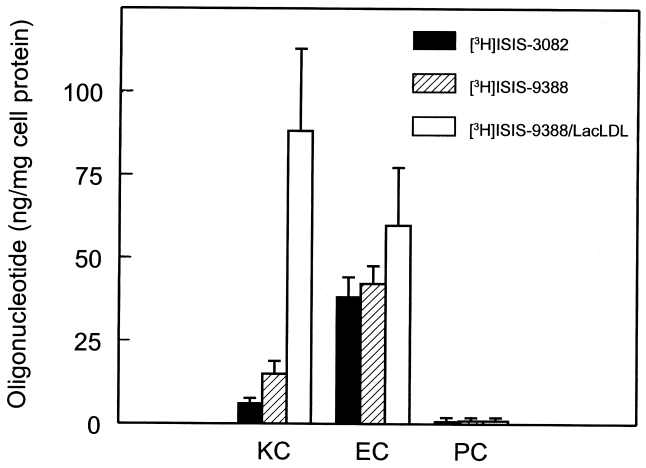

Fig. 4. Uptake of lactosylated LDL-associated ISIS-9388, free ISIS-9388, and ISIS-3082 by liver cell types. Rats were intravenously injected with [^{3}H]ISIS-3082 (■), [^{3}H]ISIS-9388 (▨), or lactosylated LDL-associated [^{3}H]ISIS-9388 (□), all at a dose of 25 μg of oligonucleotide/kg body weight. Sixty minutes later, parenchymal cells (PC), endothelial cells (EC), and Kupffer cells (KC) were isolated, and the cell-associated radioactivity was determined. The amount of oligonucleotide found in each cell type is given per mg of cell protein. Values are means ± SEM of 3–4 experiments.

4. Discussion

We show that uptake of the phosphorothioate antisense oligodeoxynucleotide ISIS-3082 by Kupffer cells is substantially increased by its conjugation to cholesterol (yield-

Table 3
Contribution of liver cell types to the liver uptake of lactosylated LDL-associated ISIS-9388, free ISIS-9388, and ISIS-3082

Cell type	Contribution to liver uptake (% of total)[a,b]		
	ISIS-9388/LacLDL	ISIS-9388	ISIS-3082
Kupffer cells	43.9 ± 5.4	14.5 ± 3.1	8.3 ± 3.2
Endothelial cells	39.4 ± 3.8	55.9 ± 7.2	62.5 ± 6.2
Parenchymal cells	16.7 ± 6.8	29.6 ± 8.1	29.1 ± 6.3

[a] The contributions of the various liver cell types to the total liver uptake of lactosylated LDL-associated [^{3}H]ISIS-9388, [^{3}H]ISIS-9388, and [^{3}H]ISIS-3082 were calculated from the data presented in Fig. 4 and the contribution of each cell type to the total liver protein.

[b] Values are means ± SEM of 3–4 experiments.

ing ISIS-9388), and subsequent incorporation of the conjugate into the lactosylated LDL carrier. ISIS-9388 associates readily with lactosylated LDL. The presence of the cholesterol moiety in the conjugate is essential, as the unconjugated ISIS-3082 does not interact with the particle. Analysis by size exclusion chromatography indicates that complexation of the lactosylated LDL particles with up to 10 molecules of ISIS-9388 does not result in the formation of aggregates. However, analysis by agarose gel electrophoresis indicates that association of increasing amounts of ISIS-9388 with lactosylated LDL leads to an increase in migration, i.e. the net negative charge, of the oligonucleotide-carrier complexes.

After intravenous injection into rats, the lactosylated LDL-associated ISIS-9388 was rapidly cleared from the circulation and taken up by the liver. At 2 min after injection, more than 50% of the dose was recovered in the liver. At 60 min after injection, when approximately 90% of the dose had been cleared from plasma, the liver contained more than 70% of the dose. Lactosylated LDL is a liver-directed carrier that is very specifically taken up by Kupffer cells, and hardly by resident macrophages in other tissue, e.g. the spleen [7]. Indeed, only a low amount ($<$1% of the dose) of lactosylated LDL-associated ISIS-9388 was recovered in the spleen, and other extrahepatic tissues contained also only small amounts of the oligonucleotide.

In the liver, Kupffer cells showed the highest uptake. These cells account for approximately 45% of the liver uptake. The reduction of liver uptake of lactosylated LDL-associated ISIS-9388 by preinjection with *N*-acetylgalactosamine indicates the involvement of galactose-specific receptors on these cells. Parenchymal cells express the galactose-specific asialoglycoprotein receptor, but the uptake of the carrier-associated oligonucleotide by these cells is very low. Kupffer cells are localized in the sinusoids, whereas parenchymal cells are situated behind a fenestrated endothelium [17]. Because of the strategic localization directly in the blood stream, lactosylated LDL is primarily taken up by the galactose-particle receptor on Kupffer cells.

Endothelial cells contribute significantly (approximately 40%) to the hepatic uptake of lactosylated LDL-associated ISIS-9388. However, we showed earlier that only a small proportion (approx. 10%) of the hepatic uptake of the lactosylated LDL carrier occurs by endothelial cells [7]. The enhanced uptake of the oligonucleotide–carrier complex by endothelial cells, compared to that of the carrier alone, has probably a dual cause. The complexes of ISIS-9388 with lactosylated LDL contained 5 oligonucleotide molecules per carrier. Agarose gel electrophoresis indicates that these complexes have an increased net negative charge. The presence of the negatively charged oligonucleotides in the surface of the lactosylated LDL particles may trigger uptake by scavenger receptors on endothelial liver cells [14,18]. Furthermore, ISIS-9388 may dissociate from its carrier after intravenous injection. In that case, the distribution of carrier-associated ISIS-9388 over liver cell types may therefore partly reflect the distribution of free ISIS-9388, which is predominantly taken up by endothelial cells. Preliminary experiments with double-labeled [^{3}H]ISIS-9388/[^{125}I]lactosylated LDL complexes indeed suggest some dissociation of ISIS-9388 from its carrier. The hepatic uptake of the ^{125}I-labeled lactosylated LDL proceeded rapidly, and after 10 min the liver contained approximately 80% of the dose. The liver accumulation of the ^{3}H-labeled oligonucleotide followed that of the carrier for 2–5 min, but then both labels diverged, and the kinetics of the liver uptake resembled that of the free oligonucleotide. The dissociation of ISIS-9388 from the carrier is likely to be due to exchange with other serum components. Phosphorothioate oligonucleotides, such as ISIS-9388, bind avidly to proteins, including serum proteins [19–21]. To prevent loss from the carrier, oligonucleotides lacking the phosphorothioate chemistry may be used. When exchange from the lactosylated LDL particle is prevented, it is expected that the carrier-associated oligonucleotide largely follows the fate of the carrier, resulting in an accumulation of oligonucleotide in the Kupffer cells that is even higher than that presently found.

Carrier-mediated delivery of phosphorothioate oligonucleotides to Kupffer cells has been reported previously. In a recent study, large oligolamellar liposomes were utilized as carriers [22]. However, the targeting of the encapsulated oligonucleotide was not very liver-specific: less than 40% of the dose was taken up by the liver and approximately 10% of the dose by the spleen (hepatic and splenic uptake of lactosylated LDL-associated oligonucleotide $>$70% and 1% of the dose, respectively).

Numerous studies have indicated that antisense oligonucleotides enter cells by endocytosis and accumulate in the endosomal–lysosomal compartment. Oligonucleotide is released from this compartment, and subsequently exerts antisense activity (reviewed in Ref. 23). Ligands that are taken up via the galactose–particle receptor also enter Kupffer cells via the endosomal–lysososmal route [7]. By associating ISIS-9388 with lactosylated LDL the influx of oligonucleotide via the endocytotic route is probably increased. The assumed increased influx leads to a higher oligonucleotide concentration in the endosomal–lysosomal compartment, which is expected to result in a proportionally higher transfer to the cytosol and higher antisense activity. Our approach has the additional advantage that a cholesteryl-conjugated oligonucleotide is utilized. The presence of a cholesterol moiety at the 3′-end of an oligonucleotide does not affect hybridization with target sequences [24], but affords protection against the action of 3′-exonucleases. This prolongs the half-life of the oligonucleotide in the endosomal–lysosomal compartment, and thus enhances its possibility to leave this compartment unharmed. Furthermore, the conjugated cholesterol is likely to facilitate passage through (lysosomal) membranes [25].

The lactosylated LDL-mediated increased uptake of oligonucleotides by Kupffer cells may be utilized to improve antisense-based therapeutic intervention during inflamma-

tion. Unconjugated phosphorothioate oligodeoxynucleotides are primarily taken up by endothelial liver cells. These cells display an enhanced expression of ICAM-1 and other adhesion molecules under inflammatory conditions, which results in the harmful infiltration of neutrophils into the liver [5,6]. Systemically administered ICAM-1-specific phosphorothioate oligonucleotides reduce the adherence of neutrophils to endothelial liver cells, and consequently exert a therapeutic effect [26]. Kupffer cells also display an increased expression of ICAM-1 during inflammation [5]. Lactosylated LDL-mediated increased uptake of ICAM-1-specific antisense oligodeoxynucleotides by the Kupffer cells is expected to down-regulate ICAM-1 expression on these cells. A lower expression of ICAM-1 on Kupffer cells will reduce or prevent the harmful infiltration of neutrophils. Furthermore, Kupffer cells produce under inflammatory conditions mediators such as tumor necrosis factor-alpha, interleukins, and prostanoids that start a cascade of events leading to serious disease [6]. Enhancement of Kupffer cell uptake of antisense oligonucleotides that abrogate the production of the inflammatory mediators may alleviate the inflammatory damage. It may be calculated that at the dose used in our study (25 μg/kg body weight), the intracellular concentration of the oligonucleotide in Kupffer cells is approximately 4 μM. This concentration has been shown to be sufficiently high to provoke a substantial down-regulation of gene expression [27,28].

In conclusion, we demonstrate in the present study that association of cholesterol-conjugated oligonucleotides with lactosylated LDL results in a substantially increased uptake of the oligonucleotide by Kupffer cells. The increased uptake of oligonucleotides by Kupffer may be utilized for the design of effective antisense-based strategies for therapeutic intervention directed at Kupffer cells.

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