Cholesterol derivative of a new triantennary cluster galactoside directs low- and high-density lipoproteins to the parenchymal liver cell

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We have developed a new triantennary galactoside, in which the terminal galactose moieties are connected to the branching point of the cluster galactoside via a 20 Å (2 nm) spacer [TG(20Å)]. *In vitro* binding studies have demonstrated that introduction of a 20 Å spacer resulted in avid and specific binding of the triantennary galactoside to the asialoglycoprotein receptor on the parenchymal liver cell. Derivatization of this galactoside with a cholesterol moiety afforded a compound [TG(20Å)C] that lowered the serum cholesterol concentration when injected into rats. In the present study we have evaluated the direct effect of TG(20Å)C on the *in vivo* fate of high-density lipoprotein (HDL) and low-density lipoprotein (LDL). A direct association of TG(20Å)C with HDL and LDL was observed on mixing these components. Incorporation of TG(20Å)C into ¹²⁵I-HDL and ¹²⁵I-LDL significantly accelerated the serum decay and concomitantly stimulated the hepatic uptake of these lipoproteins in rats. The liver uptake of TG(20Å)C-loaded ¹²⁵I-HDL or ¹²⁵I-LDL could be inhibited by 81% and 82% respectively by preinjection of 150 mg of *N*-acetylgalactosamine, indicating that

the enhanced liver uptake proceeded via galactose-specific receptors. More than 96% of the hepatic uptake of TG(20Å)C-loaded ¹²⁵I-HDL could be attributed to the parenchymal cell. Surprisingly, the parenchymal cell also accounted for 93% of the liver association of TG(20Å)C-loaded ¹²⁵I-LDL, suggesting that TG(20Å)C stimulates the uptake and processing of both lipoproteins by the asialoglycoprotein receptor on the parenchymal liver cell. This contrasts with earlier data indicating that a triantennary cluster galactoside provided with a 4 Å spacer between the terminal galactose moieties and the branching point of the dendrite stimulated hepatic uptake of LDL via the Kupffer cells. The parenchymal cell is the only liver cell type that is capable of irreversibly removing cholesterol from the body in the form of bile acids. The above results imply that administration of TG(20Å)C not only facilitates the hepatic uptake of lipoprotein-derived cholesterol (esters) but also their elimination from the body. In addition, it might be possible to utilize TG(20Å)C as a targeting device to selectively deliver large drug carriers and possibly genes to the parenchymal liver cell.

INTRODUCTION

Current therapy for hypercholesterolaemia generally aims at increasing the catabolism of low-density lipoprotein (LDL). This is achieved by up-regulating the LDL receptors on the parenchymal liver cell, which results from inhibition of cholesterol synthesis or prevention of the enterohepatic circulation of bile acids [1,2]. However, homozygotes of familial hypercholesterolaemia [3] or familial defective apo lipoprotein B₁₀₀ [4], in which the normal handling of LDL is impaired, do not always respond to this therapy [5,6]. In these cases, plasmapheresis is utilized [7]. An alternative approach for reducing high serum LDL levels might involve the removal of LDL via the galactose-recognizing asialoglycoprotein receptor on the parenchymal liver cell [8]. Theoretically, this can be accomplished using a bi-functional compound consisting of a galactoside component with high affinity for the asialoglycoprotein receptor and a component ensuring anchorage to lipoproteins.

This approach has been elaborated in our department and by others over the last few years [9–12]. A cholesterylated triantennary glycoside with terminal galactose groups [TG(4Å)C] was synthesized, which contained a lipophilic moiety to enable incorporation of the glycoside into lipoproteins [9]. This compound exerted significant hypocholesterolaemic activity in rats, establishing that it is possible to lower serum cholesterol levels by enhancing hepatic uptake via non-lipoprotein receptors [13]. However, a prolonged infusion with rather high doses of TG(4Å)C was required for therapeutic activity [13]. Furthermore,

TG(4Å)C targeted LDL to the galactose-recognizing galactose/fucose receptor on the Kupffer cell [14,15], instead of directing it to the asialoglycoprotein receptor on parenchymal cells [16]. This galactose/fucose receptor accounts for the uptake of large particles, exposing a high density of accessible galactose residues [17,18]. Recent studies indicate that an appropriate spatial arrangement of the galactose groups within a triantennary galactoside may lead to high-affinity recognition by the asialoglycoprotein receptor, even when exposed on large particles such as LDL [11,19,20]. We have now synthesized a newly devised cluster galactoside, in which the terminal galactose moieties are connected to the branching point of the dendrite by a 20 Å (2 nm) spacer [TG(20Å)]. Initial competition studies of ¹²⁵I-asialo-orosomucoid binding to the asialoglycoprotein receptor indicated that TG(20Å) has a 2000-fold higher affinity for this receptor than the galactoside from TG(4Å)C.

In the present study, the interaction of the cholesterol derivative of this high-affinity ligand, TG(20Å)C, with LDL and high-density lipoprotein (HDL), as well as its effect on the *in vivo* fate of both lipoproteins, is evaluated. Surprisingly, it is demonstrated that TG(20Å)C targets both LDL and HDL to the asialoglycoprotein receptor on the parenchymal liver cell.

MATERIALS AND METHODS

Reagents

Na¹²⁵I (carrier-free) was supplied by Amersham International, Amersham, Bucks., U.K. Collagenase type I, *N*-acetyl-D-

Abbreviations used: TG(20Å)C, *N*-[tris-*O*-(3,6,9-trioxaundecanyl-β-D-galactopyranosyl)methyl]methyl-*N*²-[1-[6-(5-cholesten-3β-yloxy)glycyl]adipyl]-glycinamide; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

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Figure 1 Chemical structures of the cholesterol-derivatized galactosides TG(4Å)C and TG(20Å)C

galactosamine and BSA (fraction V) were obtained from Sigma, St. Louis, MO, U.S.A. All other chemicals were analytical grade. The synthesis of *N*-[tris-*O*-(3,6,9-trioxaundecanyl)- β -D-galactopyranosyl]methylmethyl-*N* $^{\alpha}$ -{1-[6-(5-cholesten-3 β -yloxy)glycyl]adipyl}glycinamide [TG(20Å)C] will be described in detail elsewhere (details available on application to E.A.L.B.).

Isolation and radiolabelling of lipoproteins

Human LDL ($1.024 < d < 1.055$) and human HDL ($1.063 < d < 1.210$) were isolated from human serum by two repetitive centrifugations as described by Redgrave et al. [21]. After isolation, apoE-free HDL was obtained by chromatography on a heparin-Sepharose column [22]. LDL and HDL were radiolabelled with carrier-free ^{125}I by the ICI method of McFarlane as modified by Bilheimer et al. [23].

Formation of complexes of TG(20Å)C and LDL and HDL

TG(20Å)C, dissolved in PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl and 1 mM EDTA) (11.2 mg/ml), was mixed with radiolabelled ^{125}I -LDL or ^{125}I -HDL dissolved in PBS (final concentration of apolipoproteins 100 $\mu\text{g}/\text{ml}$). After 30 min at room temperature, the mixtures were put on ice until use. Interaction of TG(20Å)C with LDL and HDL was also studied by monitoring the electrophoretic mobility of complexes of TG(20Å)C and lipoproteins at a ratio of 0–3.0 mg of TG(20Å)C/mg of apolipoprotein using native gel electrophoresis on 0.8% agarose [24]. The ratio of TG(20Å)C to lipoprotein ranged from 0 to 285 mol of TG(20Å)C/mol for HDL and from 0 to 840 mol of TG(20Å)C/mol for LDL. After electrophoresis, protein bands were visualized by Coomassie Brilliant Blue staining.

Interaction of TG(20Å)C with lipoproteins

TG(20Å)C (200 μg dissolved in 1 ml of PBS) was incubated for 30 min at room temperature in the absence or presence of LDL (200 μg of apolipoprotein) or HDL (200 μg of apolipoprotein)

and the mixture was subjected to a density-gradient ultracentrifugation step ($1220 < d < 1006 \text{ kg}/\text{m}^3$; 150 000 g ; 22 h). After this step, the gradient was subdivided into 0.2 ml fractions and the fractions were assayed for total cholesterol and carbohydrate content.

In vivo serum clearance and hepatic uptake of lipoproteins

Male Wistar rats, weighing approximately 250–300 g, were anaesthetized by intraperitoneal injection of 15–20 mg of sodium pentobarbital. The abdomen was opened and complexes of ^{125}I -LDL or ^{125}I -HDL (50 μg of apolipoprotein in 500 μl of PBS) and TG(20Å)C, prepared as described above, were injected into the inferior vena cava. At the indicated times, blood samples of 0.2–0.3 ml were taken from the inferior vena cava. The samples were centrifuged for 2 min at 16 000 g and the serum was counted for radioactivity. The total amount of radioactivity in the serum was calculated using the equation: serum volume (ml) = $[0.0219 \times \text{body weight (g)}] + 2.66$ [25]. At the indicated times, liver lobules were tied off, excised, weighed and counted for radioactivity. The excised liver tissue amounted to less than 15% of the total liver mass. The liver uptake of the injected compound was corrected for the radioactivity in serum assumed to be entrapped in the tissue at the time of sampling (85 $\mu\text{l}/\text{g}$ fresh weight) [26].

Isolation of liver cells

Rats were anaesthetized and injected with complexes of ^{125}I -LDL or ^{125}I -HDL (50 μg of apolipoprotein in 500 μl of PBS) and TG(20Å)C as described above. After 10 min, the vena porta was cannulated and the liver was perfused with Ca^{2+} -free Hanks' balanced salt solution containing 10 mM Hepes, pH 7.4, (8 $^{\circ}\text{C}$) at a flow rate of 14 ml/min. After 8 min, a lobule was tied off in order to determine the total liver uptake. The liver was then perfused with 0.01% (w/v) collagenase at 8 $^{\circ}\text{C}$ in Hanks' solution containing 10 mM Hepes, pH 7.4, and parenchymal and non-parenchymal cells were isolated as described previously [16]. The non-parenchymal cell preparation was further fractionated into

endothelial and Kupffer cells by centrifugal elutriation as described in detail previously [27]. The contributions of the various cell types to the total liver uptake were calculated from the radioactivity recovered in the respective liver cell fractions after correction for protein content, and assuming that 92.5, 2.5 and 3.3% of the total liver protein content can be ascribed to parenchymal, Kupffer and endothelial cells respectively [27]. As found previously with other substrates [16,25,27], no significant loss of radioactivity from the cells during the isolation procedure was observed. 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, being $98 \pm 5\%$ on average.

Determination of protein, cholesterol and carbohydrate content

Protein concentrations in cell suspensions, subcellular fractions and solutions of LDL and HDL were determined by the method of Lowry et al. [28], with BSA as standard. Total cholesterol content was determined colorimetrically using the CHOD-PAP assay (Boehringer, Mannheim, Germany). Carbohydrate was determined by the phenol/ H_2SO_4 assay, as previously described by Spiro using TG(20Å)C as standard [29].

RESULTS

Interaction of TG(20Å)C with HDL and LDL

The chemical structure of TG(20Å)C is depicted in Figure 1. Introduction of a 20 Å tetraethylene glycol spacer between the galactose and the branching point of the dendrite results in a more flexible configuration of the three terminal galactose units than in TG(4Å)C. This allows more avid and specific binding of the galactoside by the asialoglycoprotein receptor (E. A. L. Biessen, H. Vietsch and T. J. C. van Berkel, unpublished work). To investigate the potential interaction of TG(20Å)C with lipoproteins, we have determined the electrophoretic pattern of HDL and LDL premixed with various amounts of TG(20Å)C. The results indicate that the electrophoretic mobility of both HDL and LDL was dose-dependently reduced at increasing concentrations of TG(20Å)C (Figure 2). The change in electrophoretic mobility of LDL and HDL on

Figure 2 Interaction of TG(20Å)C with HDL (a) and LDL (b) as determined by agarose gel electrophoresis

ApoE-free HDL (a) or human LDL (b), at a concentration of 1 mg/ml in PBS, was incubated for 30 min at room temperature with TG(20Å)C (0, 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 mg/mg of apolipoprotein, as indicated). After incubation, the complexes were subjected to native gel electrophoresis on 0.8% agarose. R_f values of HDL and LDL were 0.52 and 0.23 respectively.

Figure 3 Association of TG(20Å)C with HDL and LDL

TG(20Å)C (0.2 mg dissolved in 1 ml of PBS) was incubated for 30 min at room temperature in the absence (control) or presence of human LDL (0.2 mg of apolipoprotein) or apoE-free HDL (0.2 mg of apolipoprotein). The mixtures were subjected to density-gradient ultracentrifugation (150 000 g for 22 h at 4 °C) in NaCl/KBr/EDTA buffer (pH 7.4). The gradient was subdivided and the fractions were assayed for carbohydrate (○) and cholesterol (●) content.

incubation with TG(20Å)C suggests that efficient incorporation of TG(20Å)C into lipoproteins occurs. To quantify further the accumulation of TG(20Å)C into LDL and HDL we have monitored the density fractions of a TG(20Å)C/lipoprotein mixture, after density-gradient ultracentrifugation in an NaCl/KBr buffer, for cholesterol and carbohydrate content (Figure 3). At a ratio of TG(20Å)C to lipoprotein of 1 mg/mg, approx. 240 and 85 molecules of TG(20Å)C are incorporated into LDL and HDL respectively. In the absence of lipoprotein, TG(20Å)C was mainly recovered in the bottom fractions at high

Figure 4 Effect of TG(20Å)C on the serum decay (a) and liver association (b) of ^{125}I -HDL

TG(20Å)C was mixed with apoE-free ^{125}I -HDL (50 μg of apolipoprotein dissolved in 500 μl of PBS) at a ratio of 0 ($\circ$), 0.01 ($\blacktriangle$), 0.03 ($\blacktriangledown$), 0.1 ($\bullet$) and 0.3 ($\blacksquare$) mg of TG(20Å)C/mg of apolipoprotein. After incubation for 30 min at room temperature, the mixtures were injected intravenously into rats. At the indicated times, radioactivity in the serum (a) and liver (b) was determined. Data points are means $\pm$ S.D. of a determination performed in triplicate.

Figure 5 Effect of TG(20Å)C on serum decay (a) and hepatic uptake (b) of ^{125}I -LDL

TG(20Å)C was mixed with ^{125}I -LDL (50 μg of apolipoprotein dissolved in 500 μl of PBS) at a ratio of 0 ($\circ$), 0.1 ($\bullet$), 0.3 ($\blacksquare$) or 1.0 ($\blacklozenge$) mg of TG(20Å)C/mg of apolipoprotein. After incubation for 30 min at room temperature, the mixtures were injected into rats. At the indicated times, radioactivity in serum (a) and liver (b) was determined. Data points are means $\pm$ S.D. of a determination performed in triplicate.

densities ($d = 1200 \text{ kg/m}^3$). However, in the presence of HDL and LDL, the carbohydrate and cholesterol profile essentially coincided. Less than 14% of TG(20Å)C was recovered in the non-lipoprotein-associated form on incubation with LDL and HDL.

Effect of TG(20Å)C on the *in vivo* fate of ^{125}I -LDL or ^{125}I -HDL

The above data indicate that TG(20Å)C is efficiently incorporated

into both HDL and LDL. Subsequently, the effect of this association of TG(20Å)C with lipoproteins on the serum decay and liver association was investigated (Figure 4). At 10 min after injection of ^{125}I -HDL into rats, only $4.3 \pm 4.0\%$ of the injected dose was cleared from the circulation. The serum clearance of ^{125}I -HDL was substantially enhanced on premixing TG(20Å)C with HDL; at 10 min after injection up to $33 \pm 2.7\%$ of the injected dose was removed from the bloodstream ($P < 0.001$). Even at a dose of 0.03 mg of TG(20Å)C/mg of protein, the decay

Figure 6 Effect of preinjection of *N*-acetylgalactosamine on the hepatic uptake of TG(20Å)C-loaded ^{125}I -HDL and ^{125}I -LDL

TG(20Å)C was mixed with ^{125}I -HDL and ^{125}I -LDL (50 μg of apolipoprotein dissolved in 500 μl of PBS) at ratios of 0.3 and 1 mg/mg of apolipoprotein respectively and the mixture was incubated for 30 min at room temperature. At 1 min before the injection of the lipoprotein-TG(20Å)C complex, either solvent (■) or 150 mg of *N*-acetylgalactosamine (▲) was injected. The complexes were then injected and hepatic uptake was monitored for 40 min. As a control, hepatic uptake of native ^{125}I -HDL and ^{125}I -LDL is given (●).

of the complex in the serum was significantly accelerated compared with native ^{125}I -HDL (16.8% of the injected dose). Interestingly, the serum level of TG(20Å)C- ^{125}I -HDL complexes stabilized within 2 min of injection of the complex, suggesting a consistent but incomplete removal from the bloodstream. In agreement with the rate of serum decay, the liver uptake of native ^{125}I -HDL was low ($1.9 \pm 1.4\%$ of the injected dose). However, incubation of ^{125}I -HDL with TG(20Å)C markedly and dose-dependently stimulated the hepatic uptake of the lipoprotein to $33.9 \pm 2.6\%$ of the injected dose ($P < 0.0001$; 0.3 mg of TG(20Å)C/mg of ^{125}I -HDL). At higher ratios of TG(20Å)C to ^{125}I -HDL, stimulation of liver uptake of ^{125}I -HDL was less pronounced (results not shown).

The effect of premixing ^{125}I -LDL with TG(20Å)C on the kinetics of serum removal and liver uptake of ^{125}I -LDL were essentially similar. As with HDL, the clearance of native ^{125}I -LDL proceeded slowly (Figure 5) and only 2–3% of the injected dose was found in the liver at 10 min after injection. Preincubation with TG(20Å)C caused a dose-dependent acceleration of the clearance of ^{125}I -LDL of up to $21 \pm 2.4\%$, which was accompanied by a corresponding increase in liver association ($P < 0.001$). The maximal hepatic uptake of ^{125}I -LDL-TG(20Å)C complexes amounted to 20–25% of the injected dose at ratios of TG(20Å)C/ ^{125}I -LDL above 0.3 mg/mg.

Effect of *N*-acetylgalactosamine on TG(20Å)C-induced hepatic uptake of lipoproteins

To verify whether hepatic galactose-recognizing receptors are involved in the increased uptake of TG(20Å)C-lipoprotein complexes, we studied the effect of preinjection of *N*-acetylgalactosamine on the uptake of these complexes by liver. It appears that the hepatic uptake of both TG(20Å)C- ^{125}I -HDL and TG(20Å)C- ^{125}I -LDL was almost completely inhibited by an

excess of *N*-acetylgalactosamine (81% and 82% inhibition respectively; $P < 0.002$) (Figure 6).

Contribution of the various liver cell types to the hepatic uptake of TG(20Å)C-loaded lipoproteins

Two galactose-recognizing hepatic receptors have been identified in the liver: the asialoglycoprotein receptor on the parenchymal liver cell [8] and the galactose/fucose receptor on the Kupffer cell [14,15]. In order to identify the actual sites of cellular uptake, we determined the contribution of the various liver cell types to the TG(20Å)C-stimulated hepatic uptake of lipoproteins. The different cell types were isolated by a low-temperature procedure 10 min after the injection of TG(20Å)C-lipoprotein complexes. Parenchymal cells were responsible for at least 96% of the uptake of TG(20Å)C- ^{125}I -HDL, irrespective of the loading ratio (Figure 7). In agreement with the results in Figure 4, the absolute uptake by the parenchymal cells increased with increasing ratios of TG(20Å) to ^{125}I -HDL, reaching a maximum of 32% of the injected dose at 0.3 mg TG(20Å)C/mg of ^{125}I -HDL.

Surprisingly and in contrast with previous studies demonstrating that TG(4Å)C- and monogalactosylated cholesterol-loaded LDL were recovered mainly in the Kupffer cell fraction [16,30], the TG(20Å)C-induced increase in hepatic uptake of ^{125}I -LDL could be mainly attributed to the parenchymal liver cell as well (Figure 7). Whereas only $1.34 \pm 0.4\%$ of the injected dose of ^{125}I -LDL was recovered in the parenchymal liver cell at 10 min after injection, premixing of ^{125}I -LDL with TG(20Å)C enhanced the parenchymal cell uptake approximately 14-fold to $18.8 \pm 2.7\%$ of the injected dose ($P < 0.001$). Uptake by the endothelial and Kupffer cells was not affected by preincubation of the ^{125}I -LDL with TG(20Å)C. This means that up to 93% of the hepatic uptake of TG(20Å)C- ^{125}I -LDL (1.0 mg/mg) could be attributed to the parenchymal cell. Preinjection of *N*-acetyl-

Figure 7 Contributions of various cell types to the hepatic uptake of apoE-free HDL and LDL loaded with various amounts of TG(20Å)C

^{125}I -HDL (apoE-free, 50 μg of apolipoprotein) (□) or ^{125}I -LDL (50 μg of apolipoprotein) (▨) was mixed with 0–50 μg of TG(20Å)C in 500 μl of PBS. After 30 min at room temperature, the mixtures were injected into rats. At 10 min after the injection, parenchymal (a), endothelial (b) and Kupffer (c) cells were isolated from the liver and counted for radioactivity. Values are means $\pm$ S.E.M. of two to four experiments (performed in quadruplicate) and are expressed as percentage of the total injected dose recovered in the various liver cell types, assuming that parenchymal liver cells, endothelial cells and Kupffer cells contribute 92.5, 3.3 and 2.5% of the liver protein content respectively.

galactosamine (150 mg) or asialofetuin (15 mg), a specific ligand for the asialoglycoprotein receptor, almost completely prevented the TG(20Å)C-induced uptake of ^{125}I -LDL by the parenchymal liver cell: only 1.2 and 1.0% respectively of the injected dose of the TG(20Å)C-loaded ^{125}I -LDL was recovered in the parenchymal liver cell (results not shown). This further demonstrates that the asialoglycoprotein receptor is involved in the hepatic uptake.

DISCUSSION

We have previously developed cholesterol-lowering compounds that induce hepatic uptake of (atherogenic) lipoproteins via galactose-recognizing receptors [9,10,13,16,30–32]. Although the feasibility of this approach had been established, practical application of these compounds was hampered by their moderate hypocholesterolaemic activity. In addition, the compounds stimulated hepatic uptake of LDL by the Kupffer cells instead of the parenchymal liver cells. This is undesirable as bile acid secretion, leading to irreversible removal of cholesterol from the body, is morphologically coupled to parenchymal liver cells. It was argued that these two pitfalls might be overcome by using compounds with higher affinity and specificity for the asialoglycoprotein receptor. Previous studies on the prerequisites of ligand recognition by the asialoglycoprotein receptor on the parenchymal liver cell [17,19,20] and the galactose/fucose receptor on the Kupffer cell [15,17] have demonstrated that the asialoglycoprotein receptor preferentially recognizes tri-antennary cluster galactosides in which the terminal galactose residues possess an appropriate spatial arrangement. In order to test the above hypothesis, we have synthesized the cholesterol derivative of a highly selective cluster galactoside [TG(20Å)C], and found it to be 30-fold more effective in lowering blood cholesterol concentrations in rats on a single intravenous injection [33].

In the present study we have characterized the interaction of TG(20Å)C with LDL and HDL, and its effects on the *in vivo* fate of the lipoproteins. Incubation of lipoproteins with TG(20Å)C leads to a dose-dependent decrease in electrophoretic mobility on native agarose gels suggesting that TG(20Å)C indeed interacts

with LDL and HDL. As a consequence of incorporation of TG(20Å)C into the lipoprotein, the lipoprotein is enwrapped in a shell of oligoethylene glycols, causing a decrease in the ζ potential of the lipoprotein and thus a reduction in its electrophoretic mobility. Analysis of the density-gradient fractions of a solution of TG(20Å)C in the absence and presence of lipoprotein on density-gradient ultracentrifugation in NaCl/KBr buffer suggested almost quantitative incorporation of TG(20Å)C into LDL and HDL. At least 86% and 90% of the added TG(20Å)C was recovered in the fractions containing LDL and HDL respectively. This indicates that approximately 240 and 85 molecules of TG(20Å)C are incorporated into a single molecule of LDL and HDL respectively. Assuming that TG(20Å)C is incorporated at the surface of the lipoprotein, this corresponds to 0.15 and 0.28 molecule of TG(20Å)C per nm^2 . In comparison with TG(4Å)C, the relative amount of incorporated TG(20Å)C is only slightly lower [9]. This may be explained by the increased hydrophilicity of the compound introduced by the elongated oligoethylene glycol spacer.

A study of the *in vivo* behaviour of TG(20Å)C-lipoprotein complexes led to surprising findings. As expected, the clearance of ^{125}I -HDL from the serum was accelerated on premixing the lipoprotein with TG(20Å)C. In accordance, the hepatic uptake of ^{125}I -HDL was substantially increased, reaching a maximum of 34% of the injected dose at 0.3 mg TG(20Å)C/mg of ^{125}I -HDL. Even loading of HDL with TG(20Å)C at a ratio of 0.01 mg/mg of apolipoprotein, corresponding to 1 molecule of TG(20Å)C/molecule of HDL, resulted in a slight but significant stimulation of plasma clearance and liver uptake of the lipoprotein. Apparently, a threshold amount of TG(20Å)C is not required to enhance the asialoglycoprotein receptor-mediated hepatic uptake of TG(20Å)C-HDL complex. At ratios of TG(20Å)C/ ^{125}I -HDL higher than 0.3 mg/mg, the hepatic uptake declined to 10% of the injected dose (results not shown). As the particle size of HDL is not affected by incorporation of TG(20Å)C, this may arise from a slightly increased mobility of lipids within the HDL particle at high ratios of TG(20Å)C/ ^{125}I -HDL, facilitating the exchange of TG(20Å)C from the ^{125}I -HDL with the endogenous HDL pool. Serum decay of TG(20Å)C-lipoprotein complexes stabilizes 2 min after their injection. This

probably arises because of a rapid exchange of TG(20Å)C from the lipoprotein with endogenous lipid compartments and lipoproteins, as previously described for TG(4Å)C [16]. Kinetic studies on the rate of exchange of TG(20Å)C from ^{125}I -HDL or ^{125}I -LDL with other lipid compartments, i.e. cell membranes and lipoproteins, under *in vivo* conditions using radiolabelled TG(20Å)C are required to confirm this hypothesis conclusively. The TG(20Å)C-induced hepatic uptake of ^{125}I -HDL was found to be inhibited by 81% by preinjection with 150 mg of *N*-acetylgalactosamine, thus demonstrating that galactose-recognizing receptors are responsible for this uptake. Combined with the finding that the uptake is primarily mediated by parenchymal cells, we conclude that the asialoglycoprotein receptor is responsible for the TG(20Å)C-induced uptake of ^{125}I -HDL. This is in agreement with previous studies in which ^{125}I -HDL was targeted to the parenchymal liver cell using TG(4Å)C [32].

Premixing ^{125}I -LDL with TG(20Å)C also enhanced serum clearance and liver uptake of ^{125}I -LDL. Approx. 20–25% of the injected dose was recovered in the liver, which is lower than previously reported for TG(4Å)C and monogalactosylated cholesterol complexes [16,31]. This may be explained by the higher hydrophilicity of the compound, allowing exchange of TG(20Å)C from the premixed complex with endogenous lipid compartments. A minimal ratio of 0.1 mg of TG(20Å)C/mg of LDL (28 mol of TG(20Å)C/mol of LDL) was required for significant stimulation of hepatic uptake and serum decay of LDL. As with ^{125}I -HDL–TG(20Å)C, hepatic uptake of ^{125}I -LDL–TG(20Å)C complexes is galactose-specific and can be blocked by 82% by preinjection of 150 mg of *N*-acetylgalactosamine. In view of previous studies with TG(4Å)C [16] and monogalactosyl-cholesterol [30], it is surprising that the parenchymal liver cell rather than the Kupffer cell accounted for the bulk of the hepatic uptake of ^{125}I -LDL–TG(20Å)C complexes. Uptake by parenchymal liver cells was stimulated up to 14-fold by premixing ^{125}I -LDL with TG(20Å)C. An optimum contribution of parenchymal liver cells to hepatic uptake was found at a ratio of 1.0 mg of TG(20Å)C/mg of ^{125}I -LDL. At higher ratios, the relative contribution of the Kupffer cells increased. Kupffer cell uptake is probably mediated by the galactose/fucose receptor which preferentially recognizes large particles (> 12 nm) exposing a high density of galactose groups [17,18]. The higher affinity of TG(20Å)C than TG(4Å)C for the asialoglycoprotein receptor not only augments the hypocholesterolaemic activity of the galactoside by 30-fold but also results in a more selective delivery of particles as large as LDL to the parenchymal cell. Apparently, uptake of large galactose-exposing particles by the galactose/fucose receptor on the Kupffer cell can be avoided, provided that a high-affinity ligand for the asialoglycoprotein receptor is utilized. TG(20Å)C is therefore also an attractive targeting device for selective delivery of large drug carriers and possibly genes to the parenchymal liver cell.

In view of its potential application as a hypocholesterolaemic therapeutic, it is important that TG(20Å)C targets LDL to the parenchymal liver cell, thereby facilitating the catabolism and, eventually, the irreversible biliary removal of LDL-derived cholesterol (esters) from the body. This is confirmed by preliminary studies demonstrating that a single intravenous injection of TG(20Å)C into rats results not only in a marked reduction in serum cholesterol concentration but also in stimulation of the biliary secretion of cholesterol metabolites. As TG(20Å)C targets both the atherogenic LDL and the non-atherogenic HDL to the

parenchymal liver cell, and only irreversible removal of the former from the bloodstream is the aim in hypercholesterolaemias, preferential accumulation of TG(20Å)C into LDL will be required. Currently studies are underway to determine the actual distribution of TG(20Å)C over HDL and LDL, the most prominent lipoproteins in man.

This work was supported by the Dutch Heart Foundation (Grant 88.102).

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