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Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution

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ABSTRACT

In order to direct antisense oligonucleotides to specific tissues or cell types in vivo, we are exploring the possibility to utilize lipoproteins as transport vehicles. A 16-mer oligonucleotide (ODN) was derivatized at the 5′ prime through a ^{32}P phosphate spacer with cholesterol, yielding a ^{32}P-labeled amphiphatic cholesteryl-oligonucleotide (cholODN). Incubation of cholODN with low-density lipoprotein (LDL) for 2 hr at 37°C resulted in the formation of a cholODN-LDL complex that migrates as a single peak on agarose gel electrophoresis. The cholODN was found to bind quantitatively to both high-density lipoproteins (HDL) and LDL, but not to albumin. Stable oligonucleotide-LDL particles with up to 50 molecules of cholODN per LDL particle could be obtained. In contrast, the control ODN did not show affinity for plasma lipoproteins. Upon injection into rats, cholODN became rapidly associated with plasma lipoproteins while control ODNs were recovered in the lipoprotein deficient serum fraction. The plasma half-life of cholODN (9–11 min) is considerably prolonged as compared with the control ODN (t½ < 1 min). The cholODN-LDL was at least 5 min stable against degradation by rat plasma nucleases. It is concluded that derivatization of antisense oligonucleotides with cholesterol profoundly modifies their in vivo fate and opens possibilities for efficient and specific receptor-dependent targeting, mediated by lipoproteins coupled with specific recognition markers to various hepatic cell types.

INTRODUCTION

In many eukaryotic systems, antisense oligonucleotides (ODNs) have been used to inhibit gene expression specifically (1–3). The application of ODNs in vivo however, has been severely hampered by rapid cleavage by plasma nucleases and very short half-lives of the molecules in the blood compartment (4,5). In order to allow effective gene activity modification, a longer availability of the intact oligonucleotide and a targeted delivery is highly desirable. In the present investigation we explore the possibility to use lipoproteins as transport vehicles. Especially the low-density lipoprotein (LDL), which is responsible for the transport of 75% of the body's cholesterol, seems suitable as a carrier particle. LDL is known to bind foreign compounds like PCB's, certain steroids, benzo[a]pyrene and cyclosporin A (6–8). Association of porphyrins to LDL is thought to play an essential role in the photodynamic therapy of cancer (9,10). Molecules that associate with LDL generally show a high degree of lipophilicity, like a tris-galactoside terminated cholesterol derivative which spontaneously associates with LDL by inserting its cholesteryl moiety into the lipoprotein (11).

Based upon these properties we derivatized ODNs with cholesterol (cholODN) and investigated the association with plasma lipoproteins and the effect on the in vivo fate of ODNs. We demonstrate that: (i) both high-density lipoproteins (HDL) and low-density lipoproteins, but not albumin, bind cholODN; (ii) the cholODN is stable in rat serum for at least 5 minutes; (iii) excretion of cholODN by the kidneys is reduced 7-fold as compared with control ODN, and (iv) coupling of cholesterol to ODN prolongs its plasma half-life about 10-fold.

EXPERIMENTAL PROCEDURES

Materials

Two derivatized hexadecathymidylates (derivatized at their 5′ prime): NH_2-$(CH_2)_2$-S-S-$(CH_2)_2$-NH-5′-P*-d$(T)_{16}$ (ODN), and cholesterol-S-S-$(CH_2)_2$-NH-5′-P*d$(T)_{16}$ (cholODN) in which P* designates the ^{32}P labeled phosphate group, were synthesized as described previously by Boutorine (12). The compounds have been purified by HPLC and characterized by PAGE. ^{125}I, sodium salt (98.5% pure), was obtained from Amersham, United Kingdom. Agarose was purchased from Sigma Chemical Co., St. Louis, MO. Potassium Bromide was obtained from J.T. Baker, Deventer, the Netherlands.

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Isolation of lipoproteins

LDL was isolated from human plasma at density $1.024 < d < 1.055$ g/ml by two repetitive centrifugations according to the method of Redgrave et al (13) as described previously (11). The LDL preparation contained solely apolipoprotein B (99.97%) and no degradation products were noticeable when checked by electrophoresis in sodium dodecyl sulfate gels. Radioiodination of LDL was done according to the ^{125}I-iodine monochloride method described by Bilheimer et al (14).

Loading of LDL with oligonucleotides

For most experiments, LDL was incubated with the cholesterol-coupled oligonucleotide in a 1:5 (LDL:cholODN) molar ratio by the addition of 2−4 nmoles of cholODN in a volume of 25 µl PBS-1 mM EDTA pH 7.4 and a subsequent incubation for two hours at 37°C. Although the control oligonucleotide did not associate with LDL, all control experiments were performed with the same molar ratios (1:5) as with cholODN. For the agarose gel electrophoresis experiments presented in figures 1A and 2A, LDL was mixed with a small amount (10000 cpm) of iodinated LDL before the oligonucleotides were added.

Agarose gel electrophoresis

Aliquots of oligonucleotide-LDL complexes and control oligonucleotides were subjected to electrophoresis in agarose gels at pH 8.8 (Tris-hippuric acid buffer). After electrophoresis, the gel was cut into segments which were counted for ^{125}I radioactivity. After the addition of 5.0 ml H_2O, the segments were counted for ^{32}P activity by the method of Cerenkov in a Packard 1500 TriCarb liquid scintillation analyzer.

Redistribution of oligonucleotides to serum (lipo)proteins

Aliquots (10 µl) of oligonucleotide-LDL preparations (12.9 µg LDL) were incubated in a 200 µl solution containing 150 µg LDL, 300 µg HDL and 600 µg Human Serum Albumin. After incubation for 2 hours at 37°C, the sample was transferred to a polyallomer centrifuge tube. Thereafter, 1106 mg of solid KBr and PBS-1 mM EDTA were used for the complete dissolution of KBr to a final volume of 4.0 ml. Consecutive layers of 3.0 ml of KBr solution (1.063, 1.019 and 1.0063 g/ml, respectively) were then added. After centrifugation for 22 hours at 40,000 rpm (6×15 swing-out rotor, 4°C), 500 µl samples, starting from the bottom of the tube, were taken and the gradient was subdivided according to density. The samples were then counted for ^{32}P by the method of Cerenkov. As controls,oligonucleotide-LDL preparations were incubated with 200 µl of PBS-1 mM EDTA and analyzed as described.

Experiments *in vivo*

Male Wistar rats (280−350 g) under Nembutal anesthesia received injections of 0.15−0.3 nmoles (LDL) of oligonucleotide-LDL preparations in 500 µl PBS-1 mM EDTA, pH 7.4 in the vena cava. At regular intervals, blood samples were taken from the vena cava at least 1 cm below the site of injection,

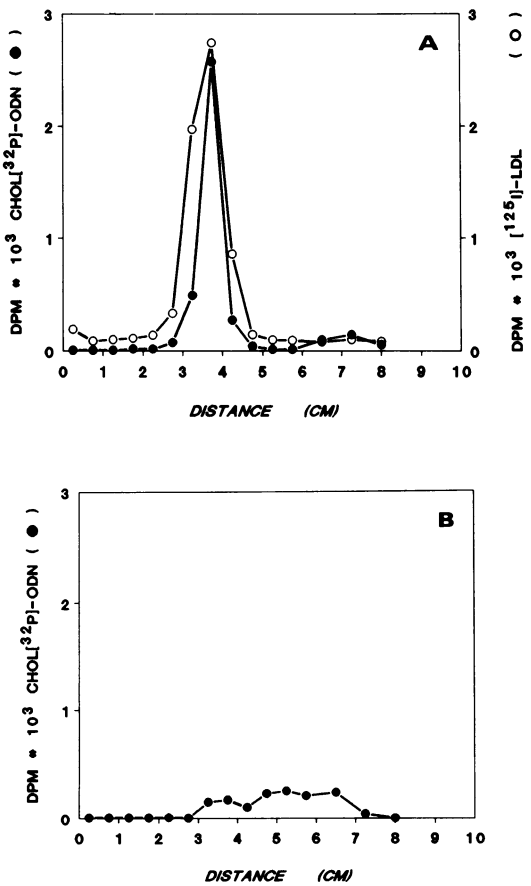

Fig. 1. Agarose gel electrophoresis of ^{32}P-cholODN incubated for 2 hours at 37°C with ^{125}I-LDL particles (A) or ^{32}P-cholODN incubated for 2 hours in the absence of LDL (B).

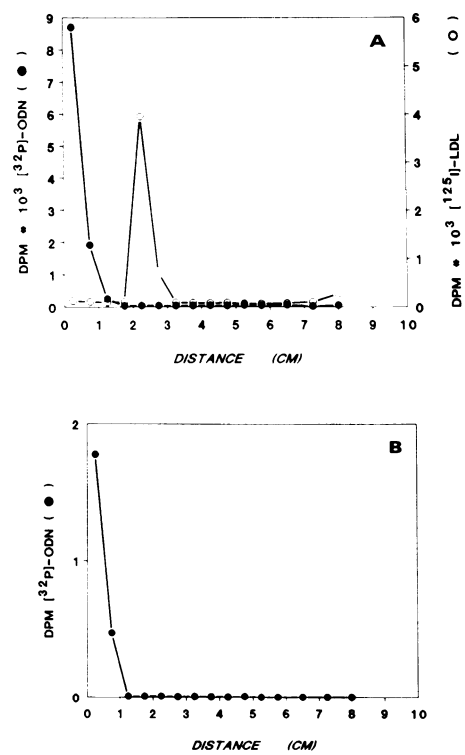

Fig. 2. Agarose gel electrophoresis of ^{32}P-ODN incubated for 2 hours at 37°C with ^{125}I-LDL particles (A) or ^{32}P-ODN incubated for 2 hours in the absence of LDL (B).

and liver lobules were excised. After 30 minutes, the animal was sacrificed and tissue samples were collected. Blood samples taken after 2 and 30 minutes were immediately stored on ice, centrifuged and plasma samples were then analyzed by density ultracentrifugation as described above. All blood samples were collected in 10 µl 100 mM EDTA and centrifuged. 150 µl plasma was mixed with 5.0 ml of HIONIC ^{R} liquid scintillation cocktail and measured for ^{32}P activity. Liver and tissue samples were weighed and homogenized in PBS-1 mM EDTA. 1.0 ml of the suspension was incubated with 30 µl H_2O_2 and 750 µl of Soluene^{R} overnight at 37°C. After the addition of 15.0 ml of HIONIC^{R} scintillation cocktail, all samples were analyzed for ^{32}P radioactivity. For liver and tissue uptake studies, all data were corrected for plasma activity (85 µl/g fresh wt (15)).

Stability of LDL-associated oligonucleotides in rat serum

27 µg (LDL protein) of LDL-oligonucleotide preparation was incubated at 37°C in 75 µl of freshly prepared serum from male Wistar rats. At regular intervals, a 15 µl sample was taken, diluted with 200 µl PBS and extracted with 200 µl phenol. After centrifugation, the aqueous layer was extracted twice with 200 µl of ether. To 175 µl of the aqueous phase 17.5 µl of 3M NaAc, 10 µg tRNA and 800 µl of EtOH was added. After incubation for 30 min at −80°C the sample was centrifuged. The supernatant was discarded and the precipitate was further dried on a SpeedVac Evaporator. The samples were then analyzed by electrophoresis using a 7M ureum-19% polyacrylamide gel which was subsequently exposed to Kodak X-OMAT AR film with an intensifying screen.

RESULTS

Association of cholODN with LDL

CholODN could be incorporated into LDL in 100% yield by incubation at 37°C for 2 hours. Agarose gel electrophoresis of ^{32}P-labeled cholODN incorporated into ^{125}I-LDL (molar ratio 5:1 = cholODN : LDL) showed that the complex migrated as a single peak with no ^{32}P activity other than at the LDL position (fig.1a). CholODN itself was not recovered at this position (fig.1b). Incubation of control ODN with LDL did not result in the formation of a drug-LDL complex (fig.2a and 2b). Although all experiments were performed with cholODN-LDL particles with a 5:1 molar ratio, it was possible to load up to 50 molecules of cholODN per LDL as revealed by agarose gel electrophoresis. 5:1 cholODN-LDL particles migrated on agarose with a relative electrophoretic mobility of 1.35 (relative to native LDL), whereas preparations with molar ratios of 50:1 had a relative electrophoretic mobility of 2.25. So, dependent on the loading grade there is a change in charge of LDL. When the cholODN complexes were examined by density ultracentrifugation, the cholODN was isolated in the fraction of density 1.019−1.063 g/ml (fig. 3a) while control ODN remained in the protein fraction of d > 1.21 g/ml (fig.4a). To examine the possible redistribution of cholODN upon contact with other lipoproteins, 12.9 µg of LDL-cholODN was incubated for 2 hours with a solution containing 150 µg LDL, 300 µg HDL and 600 µg Human Serum Albumin. This experimental setup avoided a possible degradation of the molecule since no plasma nucleases are present and allowed to assess its affinity for the different plasma components. It was

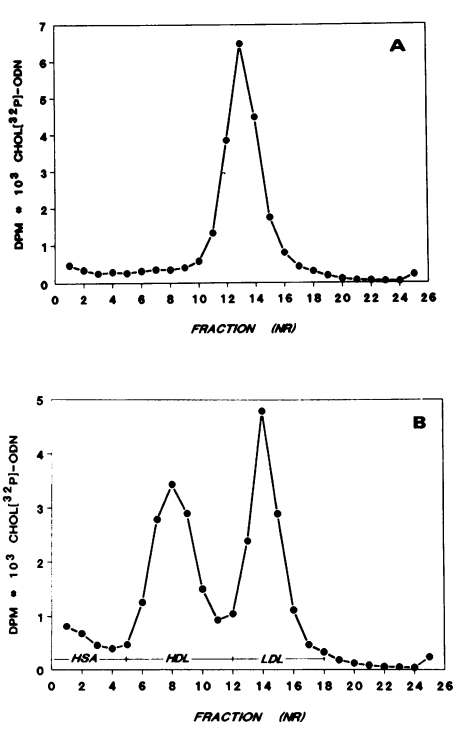

Fig. 3. Density ultracentrifugation of ^{32}P-cholODN-LDL complexes incubated for 2 h at 37°C in the absence (A) or presence (B) of a lipoprotein mixture. Preformed ^{32}P-cholODN-LDL mixtures were incubated with 150 µg LDL, 300 µg HDL and 600 µg HSA for 2 hours at 37°C (B) or with PBS-EDTA (A) and subsequently centrifuged on a KBr gradient. The fractions obtained after centrifugation correspond to the indicated lipoprotein densities.

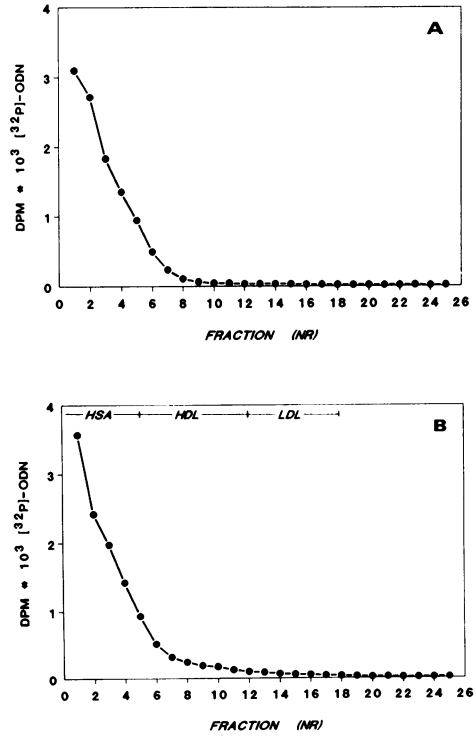

Fig. 4. Density ultracentrigugation of ^{32}P-ODN-LDL complexes incubated for 2 h at 37°C in the absence (A) or presence (B) of a lipoprotein mixture. Preformed ^{32}P-ODN-LDL mixtures were incubated with 150 µg LDL, 300 µg HDL and 600 µg HSA for 2 hours at 37°C (B) or with PBS-EDTA (A) and subsequently centrifuged on a KBr gradient. The fractions obtained after centrifugation correspond to the indicated lipoprotein densities.

found that ^{32}P activity shifted towards the HDL densities (1.063−1.21 g/ml) (fig.3b), while HSA (d > 1.21 g/ml) did not bind a significant amount. Integration of the amount of activity recovered in the HDL and LDL peaks indicated that the amount of cholODN bound was roughly proportional to the lipoprotein surface area. Control ODN, incubated with LDL was isolated at d > 1.21 g/ml (fig.4a) and incubation of the ODN-LDL with a HDL-LDL-HSA solution did not result in an increase in binding to HDL or LDL.

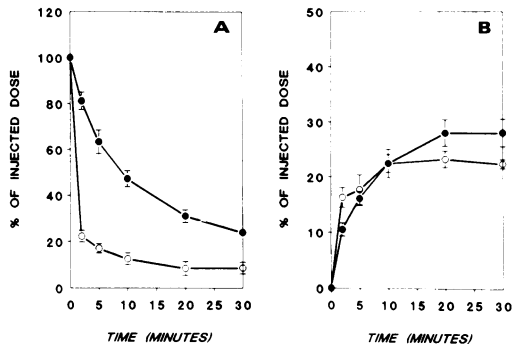

Fig. 5. Plasma decay and liver association of preincubated ^{32}P-cholODN-LDL and ^{32}P-ODN-LDL mixtures. Figure 5A, plasma decay of ^{32}P-cholODN-LDL (●) or ^{32}P-ODN-LDL (○). Figure 5B, liver uptake of ^{32}P-cholODN-LDL (●) or ^{32}P-ODN-LDL (○). Values are expressed as the mean ± S.E.M. (n=3).

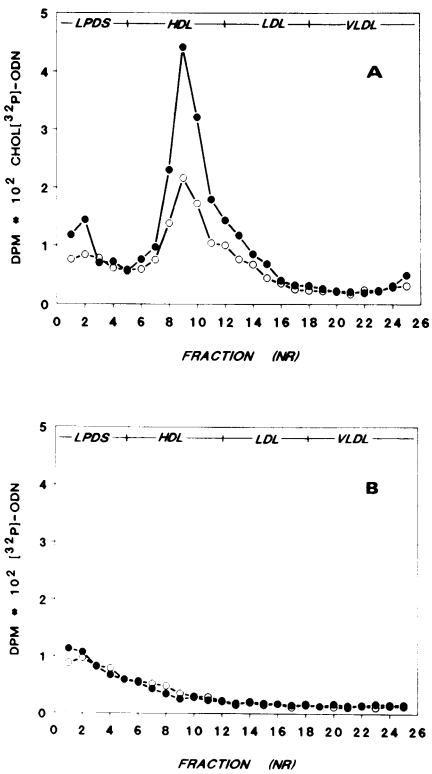

Fig. 6. Distribution of cholODN and ODN between plasma (lipo)protein fractions in vivo. The indicated preincubated (for 2 h, 37°C) mixtures were injected into rats and at the indicated times blood samples were collected. Plasma was then fractionated by density ultracentrifugation. Figure 6A, plasma distribution of ^{32}P-cholODN 2 minutes (●) or 30 minutes (○) after injection of cholODN-LDL mixtures. Figure 6B, plasma distribution of ^{32}P-ODN 2 minutes (●) or 30 minutes (○) after injection of ODN-LDL mixtures. LPDS: Lipoprotein-Deficient Serum and VLDL: Very Low-Density Lipoprotein fraction. d(LPDS)=d(HSA)>1.21 g/ml; 1.063<HDL<1.21 g/ml; 1.019<LDL<1.063 g/ml; d(VLDL)<1.019 g/ml.

in vivo fate of cholODN

As agarose gel electrophoresis and ultracentrifugation indicated that cholODN did bind to lipoproteins, we examined the effect of the association to LDL on its in vivo behavior. Even though there was no evidence for the formation of complexes between control ODNs with LDL, studies with control ODN were also performed with ODN-LDL mixtures. In figure 5, plasma decay and liver uptake of cholODN and control ODN, both preincubated with LDL, in the rat are shown. While most of the control ODN disappears from the plasma within one minute, approximately 80−90% of the cholODN is still available in the plasma at this point in time. A comparison of the rate of liver uptake of the two compounds show that the control ODN becomes associated rapidly with the liver, whereas the cholODN is taken up more gradually (fig.5b).

At 2 and 30 minutes after injection, blood samples were collected and fractionated by density ultracentrifugation in order to investigate to which plasma (lipo)proteins the compounds became associated in vivo. At two minutes after injection of cholODN, 90% of the radioactivity in the blood sample was associated with the fraction with a peak density of 1.090 g/ml, indicating that the compound is recovered mainly in the high density lipoprotein fraction. At 30 minutes, the high density lipoprotein density range is also the predominant fraction where the majority of the radioactivity is recovered (fig.6a). Both at 2 and 30 minutes after injection, the radioactivity of the control ODNs was mainly associated with the fraction of density > 1.21 g/ml. No evidence for association with lipoproteins could be found (fig. 6b). We have also examined the fate of directly injected cholODN (fig. 7). The cholODN appears to be cleared at an identical rate as cholODN that was pre-complexed with LDL. Both injection of cholODN or cholODN-LDL mixtures thus lead to a substantial increase in plasma half-life of the ODNs. We have also compared the urinary excretion of the cholODN and the control ODN. Fig 8a indicates that 30 minutes after injection, 7 times less radioactivity of the cholODN was

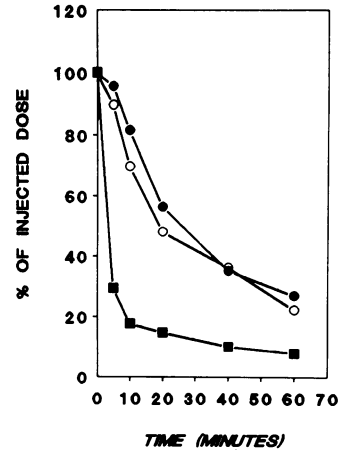

Fig. 7. Plasma decay of (chol)anti-Ras 12 mer and influence of preincubation with LDL. (○), ^{32}P-cholODN; (●), ^{32}P-cholODN-LDL and (■), ^{32}P-ODN (n=1).

associated with the kidneys relative to control ODN. Data obtained with a cholesterol-anti-Ras-ODN hybrid (that showed similar in vivo behavior as the model compound studied here), 60 minutes after injection, were in good agreement and indicated that the control ODN is processed through the kidney to the urine (fig. 8b).

Degradation of oligonucleotides in rat serum

Antisense oligonucleotides are known to be readily cleaved by nucleases (3). In order to compare the stability of the cholesterol-coupled and uncoupled ODNs, we have incubated LDL mixtures of both compounds in freshly prepared rat serum and examined the possible degradation of the oligonucleotides by subjecting them after various incubation times to gel electrophoresis followed by autoradiography. Fig. 9a shows that the cholODN remains essentially intact for 5−10 minutes in rat serum. Control ODN might be degraded slightly more rapidly than cholODN. The control ODN used in this study contains a linker moiety that already protects against degradation. With underivatized ODN we noticed more than 50% degradation within 1 minute (data not shown).

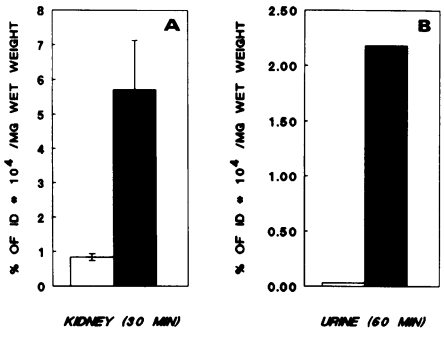

Fig. 8. The in vivo association of ^{32}P-cholODN with kidney (A) and urine (B) at various times after injection. Figure 7A, ^{32}P radioactivity of the kidney 30 minutes after injection of cholODN-LDL mixtures (white bars) or ODN-LDL mixtures (black bars). Values are expressed as the mean ± S.E.M. (n=3). Figure 7B, ^{32}P radioactivity of chol-anti-RasODN-LDL mixtures (white bars) or anti-RasODN-LDL mixtures (black bars) recovered in the urine 60 minutes after injection (n=1).

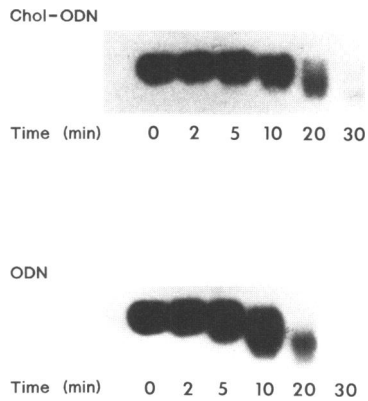

Fig. 9. Gel electrophoresis of ^{32}P-cholODN-LDL (A) and ^{32}PODN-LDL (B, ODN coupled to a linker moiety) incubated for various times in rat serum. CholODN or ODN were incubated as an LDL complex in rat serum. At regular time intervals, samples were taken and subjected to electrophoresis on 7M Urea-19% PAGE gels followed by autoradiography. Oligonucleotides and fragments varying from 16 to approximately 10 nucleotides are recovered in the gel (smaller oligonucleotides will be eluted).

DISCUSSION

Plasma lipoproteins are present in large quantities in the circulation and are responsible for the transport of hydrophobic molecules like cholesterol esters and triglycerides. Recently, some attempts have been made to utilize the capacity of lipoproteins to transport biologically active foreign compounds (16,17). In the present investigation, we have examined the interaction of a model pd(T)16 oligonucleotide and its cholesterol derivative with plasma lipoproteins, especially the low-density lipoprotein. A 2 hour incubation at 37°C of the cholODN with LDL resulted in a complete association with LDL, as indicated by agarose gel electrophoresis whereby a single ^{32}P-cholODN-^{125}I-LDL peak was observed. The control ODN exhibited no affinity for the LDL. Whereas most experiments in this study have been performed with LDL particles that contained 5 molecules of cholODN, it was possible to reach molar ratios up to 50:1. Loading LDL with increasing amounts of oligonucleotides was accompanied with a significantly enhanced electrophoretic mobility, suggesting that the cholesteryl moiety is inserted into LDL. When cholODN-LDL particles were incubated with defined HDL-LDL-Albumin solutions, a redistribution over HDL and LDL was observed. It can be calculated that the distribution of the compound between the total surface areas of HDL and LDL occurred in a 0.85:1 ratio, indicating that the compound has no specific affinity for either HDL or LDL, while albumin did not bind any cholODN. In contrast, control ODN was completely recovered in the fractions with density > 1.21 g/ml and no evidence was obtained that lipoproteins interacted with the control antisense oligonucleotides.

In vivo it was investigated to what extent the ability of cholODN to bind to lipoproteins influenced its fate. It appears that the plasma half-life, as indicated by the decay of the ^{32}P radioactivity, was increased approximately 10-fold as compared with the control ODN. The same increase was noticed when cholODN alone, without precomplexation with LDL, is injected. Radioactivity of the cholODN became associated mainly with the HDL fraction of rat plasma. It is known that in the rat, HDL is the predominant lipoprotein while only a low amount of LDL is present (18,19). It seems possible that already after 2 minutes, a redistribution of cholODN from LDL to rat HDL could have taken place, although the possibility cannot be excluded that the relative long time needed for the ultracentrifugal separation of LDL and HDL may have facilitated a further redistribution. In addition to the effect of derivatizing ODNs with cholesterol on the plasma half-life also a decreased urinary excretion is noticed. This property is expected to be beneficial for increasing the exposure of the body to antisense oligonucleotides.

Oligonucleotides are known to be subject to degradation by nucleases (3). An examination of the stability of cholODN-LDL particles in rat plasma indicated that cholODN was recovered unchanged after exposure to rat serum for 5−10 minutes. Therefore, for cholODN and ODNs which are present in the circulation longer than this time period, at least a partial degradation had occurred. In the initial phase, however, the oligonucleotides are still intact. As the 5′ prime end of the oligonucleotide is derivatized with cholesterol, which forms the

anchor in LDL, it can be argued that the 5′ prime of the compound is stabilized, leaving the 3′ prime end as major target for degradation. Currently, we are developing ways to improve the stability of ODN further by protecting the 3′ prime end. However, it can be concluded from the present data that, considering the increased half-life and plasma stability of the present cholODN, this molecule already offers possibilities for an induced specific uptake in organs like the liver. Well-defined (modified) lipoprotein recognition systems have been described (20) that are coupled to uptake of the particles within 2 to 3 minutes after injection. As the studied cholODN is still intact at this point in time, it can be argued that undegraded oligonucleotides can be delivered in vivo via these rapid uptake pathways. Association of cholesterol-linked antisense oligonucleotides with these carrier systems may lead to an effective targeting to the liver whereby various hepatic disorders linked to abnormal activation of certain genes might be treated specifically and efficiently.

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