Liver Uptake of Phosphodiester Oligodeoxynucleotides Is Mediated by Scavenger Receptors

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ABSTRACT
The therapeutic activity of antisense oligodeoxynucleotides (ODNs) often is impaired due to premature degradation and poor ability to reach the (intracellular) target. In this study, we addressed the in vivo fate of ODNs and characterized the major sites responsible for the clearance of intravenously injected phosphodiester ODN. On injection into rats, 32P-ODNs (mischelaneous sequences and GT-containing ODNs with variable G content) are rapidly cleared from the bloodstream (t1/2 = 0.6–0.7 min), with the liver being the main site of elimination. The contribution of the liver to ODN clearance depended on its sequence and varied considerably. Hepatic uptake tended to be lower for G-rich ODNs as a result of increased bone marrow uptake. Within the liver, both Kupffer cells (KC) and endothelial cells (EC) were responsible for 32P-ODN uptake. To elucidate the mechanism of liver uptake, 32P-ODN binding studies using isolated EC and KC were performed. Binding to both cell types seemed to be saturable, of moderate affinity, and mediated by a membrane-bound protein. The inhibition profiles of 32P-ODN binding to EC and KC by various (poly)anions were essentially equal and corresponded closely to those of 125I-acetylated low-density lipoprotein. In summary, the results indicate that scavenger receptors on nonparenchymal liver and bone marrow cells contribute to the elimination of ODNs from the bloodstream. Minor changes in ODN sequence markedly affect receptor recognition, resulting in considerable shifts in the biodistribution of antisense ODNs.

Antisense ODNs have been shown to interdict gene expression at various levels (Stein and Cheng, 1993). Because their inhibitory activity is highly specific and their action rarely accompanied by toxic side effects, ODNs form promising clinical alternatives for conventional drug therapy. For effective therapeutic application, the pharmacokinetics of ODNs must be mapped thoroughly. The in vivo fate of ODNs and DNA in animal models has been addressed in a number of studies (for a review, see Crooke and Bennett, 1996). ODNs, and phosphodiester ODNs in particular, were reported to be cleared rapidly from the bloodstream after injection into rats due to extensive liver and kidney uptake (Emlen et al., 1988; De Smidt et al., 1991; Zendegui et al., 1992; Inagaki et al., 1994; Sands et al., 1994; Rafi at al., 1996; Sawai et al., 1996). The qualitative studies of Emlen et al. (1988) and Rafi at al. (1996) suggested that within the liver, nonparenchymal liver cells were responsible for hepatic uptake of double-strand DNA and phosphorothioate ODNs, respectively. Although the renal disposition of ODNs was claimed to be mediated by scavenger receptor A-VII (Sawai et al., 1996), the hepatic recognition sites responsible for the ODN uptake remain to be identified.

On the basis of in vitro studies, a number of cellular uptake pathways for ODNs and DNA have been suggested (Bennet et al., 1985; Loke et al., 1989; Yabukov et al., 1989; Pearson et al., 1993; Stein et al., 1993; Zhao et al., 1993; Benimetskaya et al., 1997). Bennett et al. (1985) showed that leukocytoblasts bind and take up λ-phage DNA via intracytoplasmic vacuoles through a 30-kDa receptor. Yabukov et al. (1989) and Stein et al. (1993) characterized the mechanism of binding and uptake of 32P-ODN by fibroblasts and HL-60 cells. The uptake of ODN seemed to be mediated in part by pinocytosis (Stein et al., 1993) and in part by MAC-1 (CD11b/CD18; Benimetskaya et al., 1997). In addition, Pearson et al. (1993) recently demonstrated that 32P-ODN uptake was increased in scavenger receptor-transfected CHO cells. Together with the finding that scavenger receptor-expressing nonparenchymal liver cells contribute to hepatic uptake (Emlen et al., 1988), this is suggestive of an important role for scavenger receptors in ODN clearance in vivo.

Despite the fact that the above studies indicate that different and cell-specific pathways may be implicated in uptake of ODNs, the actual nature of the elimination sites of the major elimination site, the liver, is still under investigation. We

ABBREVIATIONS: ODN, oligodeoxynucleotide(s); DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EC, endothelial cell(s); KC, Kupffer cell(s); AcLDL, acetylated low-density lipoprotein; OxLDL, oxidized low-density lipoprotein; PAGE, polyacrylamide gel electrophoresis; LDL, low-density lipoprotein.
therefore have mapped the pathway or pathways for hepatic uptake of ODNs in greater detail so ODNN delivery protocols can be designed that either use or avoid these recognition systems.

**Experimental Procedures**

**Materials.** Na$^{125}$I in 0.1 M NaOH (13.5 mCi/μg) and [γ$^{32}$P]ATP was purchased from Amersham (Buckinghamshire, England). Collagenase (type IV), trypsin blue, BSA (fraction V), levanoside HCl, chloroquine diphosphate, polyadenosinic acid (potassium salt), polyguanosinic acid (potassium salt), polyctydic acid (potassium salt), heparin (from porcine intestinal mucosa, grade I), and ficoidin (from *Fucus vesiculosus*) were purchased from Sigma Chemical (St. Louis, MO). Salmon sperm DNA (native; molecular mass, 3.8 × 10⁶ Da) and T4-poly nucleotide kinase were purchased from Pharmacia (Woerden, The Netherlands). Monensin (sodium salt) was obtained from Calbiochem-Behring (La Jolla, CA). ODNN were synthesized at the Department of Organic Chemistry (Dr. J.H. Van Boom) (5′-GAC.TT-T.AGT.CGT.CGT.GGA-cap, ODNN1; 5′-TCC.ACG.ACG.ACT.AAA.GTC.TTT-cap, ODNN2) or purchased from Eurogentec (Seraing, Belgium) (GTG.CCG.GGG.TCT.TGG.GGC-cap, ODNN3; TTT.TTT.TTG. TTG.TTT.T, dG₁₆₅, TGG.TGG.TGG.TGG,T, dG₁₆₆₉, GGG.TGG.GTG.GGT. GGG.G, dG₁₆₇, GGG.GGG.GGG.GGG.GGG.G, dG₁₇₆).

**In vivo serum clearance and liver association.** Male Wistar rats, weighing ~250–300 g, were anesthetized by intraperitoneal injection of 15–20 mg of sodium pentobarbital. The abdomen was opened, and $^{32}$P-ODN (10 μg in 500 μl of PBS (10 mM Na₂PO₄, 150 mM NaCl, pH 7.4)) was 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 serum was calculated using the equation: serum volume (ml) = [0.0219 × body weight (g)] + 2.66 (Bijsterbosch et al., 1989). At the indicated times, liver lobules were tied off, excised, weighed, and counted for radioactivity. The total excised liver tissue amounted to <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 μl/g wet weight) (Caster et al., 1955).

**Isolation of liver cells.** Rats were anesthetized and injected with $^{32}$P-ODN (10 μg in 500 μl of PBS) as described. At 10 min after the injection, the vena portae was cannulated, and the liver was perfused with Ca$^{++}$-free Hanks’ balanced salt solution plus 10 mM HEPES, pH 7.4 (<8°), at a flow rate of 14 ml/min. After 8 min, a lobule was tied off, and the liver was perfused with 2% (w/v) BSA and 0.2% (v/v) BSA at 4°. After incubation, the lobules were washed twice with DMEM plus 0.2% BSA and once with DMEM, and the cell-associated radioactivity was counted. Nonspecific binding was defined as the binding of $^{32}$P-ODN1 in the presence of 100 μg/ml polyinosinic acid.

For competition studies, KC and EC were incubated with $^{32}$P-ODN1 (20 nM) or $^{125}$I-AcLDL (5 μl/g, 9.6 nM) and displacer for 2 hr at 4°. After incubation, cells were processed as described. Cell-bound radioactivity was determined and corrected for protein content. During incubation at 4° with EC or KC, $^{32}$P-ODN1 seemed to be stable for 2 hr as determined with PAGE analysis of the $^{32}$P-ODN1.

**Kinetics of initial uptake of $^{32}$P-ODN1 by EC and KC.** Uptake of $^{32}$P-ODN1 by EC and KC was studied as described. KC (1.5 × 10⁶; 150 μg of cell protein) or EC (2 × 10⁶; 150 μg of cell protein) in DMEM (0.5 ml) containing 2% (w/v) BSA were incubated for 2 hr at 4° with a concentration of radiolabeled ligand of 5–250 nM. After incubation, cells were washed twice with DMEM plus 0.2% BSA and once with DMEM, and the cell-associated radioactivity was counted. Nonspecific binding was defined as the binding of $^{32}$P-ODN1 in the presence of 100 μg/ml polyinosinic acid.

**Data processing.** Saturation binding data and substrate curves of initial uptake were analyzed according to a single-site binding model using nonlinear regression (GraphPAD, ISIS Software). Displacement studies were analyzed according to a single-site competitive displacement model using nonlinear regression (Bijsterbosch et al., 1988). Statistical significance of the differences was quantified by Student’s *t* test.
Results

The in vivo behavior of three phosphodiester ODNs (miscellaneous sequences with G contents of 16–50%) has been addressed in rats. As a measure for the stability of ODNs in the circulation, we first determined the rate of degradation of $^{32}$P-ODN1 in the presence of serum (50% v/v) at 37°. ODN1 is degraded slowly in the presence of serum at an apparent half-life of 19 ± 6 min (data not shown). On intravenous injection into rats, the miscellaneous ODN sequences (ODN1, ODN2, and ODN3) were cleared rapidly from the bloodstream with similar elimination half-lives of ~0.65 min (Fig. 1). Within 2 min after injection, only 11.2–12.9% of the injected dose resided in the serum. At this time, <10% of $^{32}$P-ODN1 is degraded as determined by PAGE. The liver seemed to be the main site of clearance: $^{32}$P-ODN1 (G content, 33%) displayed the highest uptake (36 ± 4% of the injected dose), whereas liver uptake of $^{32}$P-ODN2 (16% G content) and $^{32}$P-ODN3 (an antisense sequence for murine c-myb; 50% G content) amounted to 22 ± 2% and 23 ± 2%, respectively. Other major sites of ODN recovery were skin (recovery ranging from 9.0–13.7% of the injected dose for the various ODNs), muscles (8.8–13.8%), small intestine (5.0–7.5%), kidneys (1.3–3.5%), and bone marrow (7.0–13.4%). Because liver uptake varied considerably from one sequence to another and because it has been suggested that receptor recognition may be influenced by the G content (Pearson et al., 1993), we assessed the effect of G content on the in vivo fate of GT-containing model ODNs. These GT-containing ODNs are telomere-like oligodeoxyribonucleotides that have the tendency to form quadruplex structures at high G contents. These quadruplexes are very good substrates for SR-AI/II (Pearson et al., 1993). The serum decay of these GT-ODNs was essentially similar to that of the miscellaneous sequences. Liver uptake was maximal for dG$_{10}$T$_6$ (G content, 62%; 31 ± 2% liver uptake). At higher G contents [i.e., dG$_{13}$T$_3$ (81%) and dG$_{16}$ (100%)], liver uptake was strongly impaired due to a significantly increased bone marrow uptake (up to 45% of the injected dose for dG$_{16}$). Hepatic uptake of $^{32}$P-ODN1 was significantly reduced after heat-induced denaturation (22 ± 2% versus 36 ± 4% for control ODN1; p < 0.01). Isolation of the various liver cell types at 10 min after injection revealed that hepatic ODN uptake could be mainly ascribed to KC (39% average) and EC (43%) (Fig. 2). Parenchymal liver cells, although constituting 92.5% of the total liver mass, were responsible for only 13–22% of the liver uptake. There was no direct correlation between the ratio of EC to KC uptake and the G content or sequence of the ODN.

To identify the recognition site or sites responsible for liver uptake, we investigated the interaction of the ODN with the highest liver uptake, ODN1, with isolated EC and KC. To establish conditions for equilibrium binding, we first determined the kinetics of the association of $^{32}$P-ODN1 to EC and KC showing that at 4° and 1 nM $^{32}$P-ODN1, equilibrium binding was achieved within 2 hr of incubation. $^{32}$P-ODN1 binding to EC and KC was monophasic (Hill coefficient close to unity), saturable ($B_{max} = 112.5 ± 4.9$ and $51.9 ± 5.2$ ng/mg, respectively), and of moderate affinity ($K_i = 109 ± 22$ and $102 ± 9$ nm, respectively) (Fig. 3). The protein nature of the $^{32}$P-ODN1 binding site or sites on both cell types was established by determining the effect of pretreatment of the cells with trypsin (37° for 15 min). Because binding to EC and KC was reduced by 60–80% (Fig. 4), it can be concluded that $^{32}$P-ODN1 binding is largely mediated by a membrane-bound protein. Incubation of the cells with glycine buffer (pH 2.8) also impaired binding by 70%, confirming that a protein receptor may be involved in $^{32}$P-ODN1 binding to both cell types.

Fig. 1. Liver uptake (top) and serum half-life (bottom) of various $^{32}$P-ODNs in the rat. $^{32}$P-ODNs (ODN1, ODN2, ODN3, dG$_{10}$T$_6$, dG$_{14}$T$_6$, and dG$_{16}$; 4 µg) were injected intravenously into rats. At 1, 2, 5, 10, 20, 30, and 40 min after injection, radioactivities in serum were determined, and the serum half-life was calculated using a computerized single-phase exponential decay algorithm. Liver-associated radioactivities was determined at 40 min after injection. Values are mean ± standard deviation of a determination performed in triplicate (ODN1 and ODN2) or duplicate (ODN3, dG$_{14}$T$_6$, dG$_{14}$T$_6$, and dG$_{16}$).

Fig. 2. Contribution of various cell types to the liver association of $^{32}$P-ODN1. $^{32}$P-labeled ODN (ODN1, dG$_{13}$T$_3$, dG$_{16}$T$_6$, dG$_{16}$T$_6$, and dG$_{16}$; 10 µg in 500 µl of PBS) was injected into rats. At 10 min after injection, parenchymal cells (PC), EC, and KC were isolated from the liver, and cellular radioactivity was counted. Values are mean ± standard deviation of three (ODN1) or two (other ODNs) experiments and are expressed as percentage of the total injected dose that is recovered in the various liver cell types, assuming the parenchymal liver cell, EC, and KC contribute 92.5%, 3.3%, and 2.5% to the liver protein content, respectively.
Interaction of Oligodeoxynucleotides with Liver Cells

Fig. 3. Left, $^{32}$P-ODN1 binding to EC (■) and KC (■). Effect of trypsin digestion. EC or KC (2–3 × 10⁶ cells) were incubated for 15 min at 37°C in 1 ml of DMEM or trypsin (500 µg/ml) and subsequently washed thoroughly with DMEM supplemented with 2% BSA. Then, cells were incubated for 2 hr at 4°C with $^{32}$P-ODN1 (20 nM). After incubation, cells were immediately washed as described in Experimental Procedures. Cell-bound radioactivity was determined and corrected for protein content (100% binding corresponds with 14.2 ng/mg for EC and 4.5 ng/mg for KC). Right, Effect of pH 2.8 shock or EDTA treatment. EC or KC (2–3 × 10⁶ cells/ml) in 1 ml of DMEM were incubated for 2 hr at 4°C with 20 mM $^{32}$P-ODN1 in the absence or presence of EDTA (10 mM). After incubation, cells were either washed as described above or incubated with glycine buffer (200 mM, pH 2.8) for 10 min at 4°C and subsequently washed. Cell-bound radioactivity was determined and corrected for protein content.

Fig. 4. Saturation curve of $^{32}$P-ODN1 binding to EC and KC at 4°C. EC (○) or KC (□) (2–3 × 10⁶ cells/ml) were incubated for 2 hr at 4°C with 0–250 nM $^{32}$P-ODN1 in the absence (○) or presence (□) of 100 µg of polyinosinic acid. After incubation, the cells were washed thoroughly, and the cell-associated radioactivity was determined. Inset, Scatchard plots of the two binding curves are given (○, EC; □, KC). Lines, nonlinear regression analysis of the binding curves.

Fig. 5. Effect of various (poly)anions on the binding of $^{32}$P-ODN1 (closed bars) or $^{125}$I-AcLDL (open bars) to EC (top) and KC (bottom). EC or KC (2–3 × 10⁶ cells/ml) were incubated in DMEM (supplemented with 2% BSA), with $^{32}$P-ODN1 (20 nM) or $^{125}$I-AcLDL (9.6 nM), in the absence (control) or presence of polyinosinic acid (polyI, 100 µg/ml), polyadenosinic acid (polyA, 100 µg/ml), polyguadinilic acid (polyG, 100 µg/ml), polyctydinidic acid (polyC, 100 µg/ml), AMP (50 µM), ADP (50 µM), ATP (50 µM), dG₃T₆ (1 µM), dG₆T₃ (1 µM), dG₆T₉ (1 µM), lipopolysaccharide (LPS) (150 µg/ml), heparin (100 units/ml), salmon sperm DNA (ssDNA) (300 µg/ml), or fucoidin (100 µg/ml) for 2 hr at 4°C. After incubation, cells were washed thoroughly, and the cell-bound radioactivity was determined. Radioligand binding is plotted as percentage of total binding (in the absence of displacer) and is the mean of a duplicate determination (100% binding corresponds with 14.2 ± 1.5 ng/mg for EC and 7.1 ± 1.0 ng/mg for KC).

$^{32}$P-ODN1 binding. Lipopolysaccharide (150 µg/ml) and heparin (100 units/ml) were moderately potent, whereas salmon sperm DNA (500 µg/ml) was completely ineffective as an inhibitor. The inhibition profiles of $^{32}$P-ODN1 binding to EC and KC not only were mutually identical but also closely matched that of $^{125}$I-AcLDL binding to both cell types, suggesting that scavenger receptor type binding sites are involved in $^{32}$P-ODN1 binding to nonparenchymal liver cells. Therefore, we monitored the effect of AcLDL and OxLDL, both established substrates for the most relevant scavenger receptors [scavenger receptor class A-I/II (Horiuchi et al., 1985), CD36 (Endeman et al., 1993), and Fcγ/RII (Stanton et al., 1992)] on $^{32}$P-ODN1 binding (Fig. 6). AcLDL seemed to reduce $^{32}$P-ODN1 binding to EC and KC by ~40% (six experiments), whereas OxLDL led to a small but significant 15–
20% inhibition; however, there was a considerable interexperimental variation in the effect of both modified lipoproteins on 32P-ODN binding.

The efficacy of two ODNs (ODN1 and ODN3), the polyanion polyinosinic acid, and the mononucleotide ADP to displace 32P-ODN binding to EC and KC was studied in more detail using competition binding studies (Fig. 7). All compounds fully inhibited specific 32P-ODN1 binding in a competitive fashion with inhibition constants of 74 ± 23 nM (unlabeled ODN1), 10 ± 3 nM (ODN3), 0.052 ± 0.008 nM (polyinosinic acid), and 31 ± 7 mM (ADP) for EC and 68 ± 16 nM (unlabeled ODN1), 38 ± 6 nM (ODN3), 0.036 ± 0.004 nM (polyinosinic acid), and 27.1 ± 4.4 mM (ADP) for KC.

To verify whether the 32P-ODN1 binding site or sites also mediate ligand internalization, we determined uptake at 37° (i.e., total cell-associated radioactivity after removal of the membrane-bound 32P-ODN1 by treatment with 100 mg/ml polyinosinic acid). Uptake proceeded linearly in time for 10–15 min and leveled off after 60 min of incubation (data not shown). Hence, a 10-min incubation time was selected for uptake studies (unless otherwise stated). The stability of 32P-ODN1 in the presence of EC and KC at 37° was sufficient to allow a 10-min incubation with both cell types (half-lives of EC and KC, 48 ± 7 and 55 ± 7 min, respectively). Uptake of 32P-ODN1 followed monophasic Michaelis-Menten kinetics with a $K_m$ value of 270 ± 21 and 106 ± 64 nM for EC and KC, respectively, and was inhibited for 80–90% in the presence of 100 µg/ml polyinosinic acid. Apparently, 32P-ODN1 is inter-
nalized efficiently after initial binding. To characterize the pathway of $^{32}$P-ODN1 uptake, we studied the effect of various agents that interfere with lysosomal uptake on uptake of $^{32}$P-ODN1 (Fig. 8A). Sodium azide (84 ± 4% and 77 ± 4%, respectively), sucrose (74 ± 2% and 63 ± 3%), monensin (70 ± 7% and 63 ± 4%), and chloroquine (86 ± 4% and 71 ± 8%) significantly reduced uptake of $^{32}$P-ODN1 by EC and KC. Although these results demonstrate that uptake of $^{32}$P-ODN1 proceeds via an energy-dependent uptake mechanism, they leave unresolved whether $^{32}$P-ODN1 enters the cell intact or as an metabolite formed after extracellular degradation of membrane-bound $^{32}$P-ODN1. The effects of phosphomonoesterase, phosphodiesterase, and phosphatase inhibitors on the rate of uptake were determined to discriminate between these two pathways (Fig. 8B). GMP, an analogue of the 5’-terminal nucleoside (thus inhibiting receptor-mediated uptake of exonuclease-digested $^{32}$P-ODN1 metabolites); 4-nitrophenyl phosphate, a phosphomonoesterase inhibitor; L-(-)-tartaric acid, an acid phosphatase inhibitor; levasimole, an alkaline phosphatase inhibitor; and a mixture of these agents had no effect on $^{32}$P-ODN1 uptake. Only 4-nitrophenyl phosphate tended to enhance $^{32}$P-ODN1 uptake by EC, probably by increasing the stability of $^{32}$P-ODN1 in the incubation buffer.

Finally, we analyzed the cell lysates of EC and KC, which had have been incubated for 0–30 min at 37°C with $^{32}$P-ODN1, on PAGE for the presence of intact $^{32}$P-ODN1 (Fig. 9). In both cell types, intact $^{32}$P-ODN1 could be detected in the lysosomal compartment, and the intracellular amount of intact $^{32}$P-ODN1 in both cell types increased in time after a 2-min lag phase, reaching a maximum at 20 min of incubation at 37°C. Degradation products of $^{32}$P-ODN1 were not observed during this time course.

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**Fig. 9.** PAGE analysis of the kinetics of $^{32}$P-ODN1 internalization by EC and KC. EC or KC (2–3 × 10^6 cells/ml) were incubated in DMEM (supplemented with 2% BSA) with 20 nM $^{32}$P-ODN1 for 0, 1, 2, 5, 10, 20, or 30 min at 37°C. After incubation, the cells were put on ice, washed once with ice-cold DMEM (1 ml; supplemented with 2% BSA), and incubated for 5 min at 4°C with polyniosinic acid (100 μg/ml) in DMEM plus 2% BSA) to remove membrane-bound ODN1. Subsequently, the cells were heated for 5 min at 96°C, and sonicated, after which $^{32}$P-ODN1 was isolated from the cell lysate by phenol/chloroform extraction. The isolated $^{32}$P-ODN1 was subjected to gel electrophoresis on 19% polyacrylamide gel under denatured conditions (80 mM Tris/90 mM boric acid/2 mM EDTA/7.5 M urea), and the gel was autoradiographed using the Phosphor-Imager.
was monitored using displacement studies. Of the tested polyanions, only unlabeled ODN1, polyinosinic acid, polyguanosinic acid, and fucoidin inhibited $^{32}\text{P}$-ODN1 binding, whereas structural analogues like polyadenosinic and polycytidinic acid were not capable of displacing $^{32}\text{P}$-ODN1 binding. In concert with the data of Pearson et al. (1993), the ability of 16-mer to displace $^{32}\text{P}$-ODN binding increased with increasing G content. Of the other anions, only nucleotide triphosphates, lipopolysaccharide (Hampton et al., 1991), and fucoidin inhibited ODN binding. This illustrates that recognition of polyanions is a highly specific process.

Three main conclusions can be drawn from the competition studies. First, the binding profiles of EC and KC are essentially equal, making it likely that the $^{32}\text{P}$-ODN1 recognition sites on both cell types are identical. Second, the binding characteristics of $^{32}\text{P}$-ODN1 binding to EC and KC do not concur with that of p80, which is involved in ODN binding to HL-60 cells, or with the ODN receptor described by Yabukov et al. (1989). Binding of $^{32}\text{P}$-ODN to HL-60 cells seemed to be inhibitable by nucleotide monophosphates and tRNA (Loke et al., 1989), whereas we did not observe any effect of these inhibitors. Moreover, double-stranded DNA, which is an efficient inhibitor of ODN binding to the receptor of Yabukov et al. on fibroblasts, is unable to displace $^{32}\text{P}$-ODN1 binding to nonparenchymal liver cells. Third, the characteristics of $^{32}\text{P}$-ODN1 binding to EC and KC closely parallel that of $^{125}\text{I}$-AcLDL binding to these cell types. Recent studies have shown that a number of scavenger receptors are able to take up $^{125}\text{I}$-AcLDL, including scavenger receptor class A-I/II (Horiiuchi et al., 1985; Ashkenas et al., 1993; Doi et al., 1993; Pearson et al., 1993; De Rijke et al., 1994), CD36 (Endeman et al., 1993), the FcγRII receptor (Stanton et al., 1992), and other still-unidentified anion receptors (Suzuki et al., 1997). In fact, Suzuki et al. (1997) demonstrated that the in vivo fate of AcLDL in scavenger receptor AI/II-deficient mice was equal to that in control mice. This suggests that in addition to scavenger receptor class AI/II, alternative receptors exist in the liver. Of the potential candidate scavenger receptors for mediating ODN uptake by nonparenchymal liver cells, CD36 can be excluded because ligand binding to CD36 is not inhibitable by polyinosinic acid (Endeman et al., 1993). The in vivo data presented in this study confirm the in vivo finding that multiple receptors may contribute in ODN clearance. Excess AcLDL and OxLDL (being good substrates for scavenger receptor AI/II and CD36) only partly inhibit $^{32}\text{P}$-ODN1 binding to EC and KC, whereas polyinosinic acid completely inhibits binding.

In addition, the ODN binding capacities of EC and KC (i.e., 18.8 and 9.0 pmol/mg of cell protein, respectively) greatly exceed that of $^{125}\text{I}$-AcLDL binding to both cell types (271 ± 33 and 58 ± 19 fmol/mg of cell protein, respectively; three experiments; data not shown).

Uptake studies at 37° demonstrated that $^{32}\text{P}$-ODN1 binding to EC and KC is followed by internalization. At least 54% (KC; turnover rate, 18 min) and 89% (EC; turnover rate, 11 min) of the membrane-bound ODN was internalized within 10 min at 37°, which is slightly slower than that of $^{125}\text{I}$-AcLDL by both cell types (Van Berkel et al., 1981; Nagelkerke et al., 1983). Internalization (which includes both uptake and initial degradation) was markedly impaired after energy depletion of EC and KC (sodium azide), inhibition of membrane clustering (succrose), or inhibition of lysosomal acidification (chloroquine and monensin). This is in agreement with previous studies of Van Berkel et al. (1981) and Nagelkerke et al. (1983) showing that AcLDL degradation (≥90%) and, to a lesser extent, uptake (≥40%) is inhibited in the presence of lysosomotropic agents such as chloroquine. Apparently, the segregation of scavenger receptors and associated substrates is slightly affected in the presence of these agents. Neither phosphodiesterase, phosphomonoesterase, nor phosphatase inhibitors nor a mixture of these agents significantly affected ODN uptake, suggesting that intact $^{32}\text{P}$-ODN1 is internalized. This is confirmed by autoradiographic analysis of EC and KC cell lysates, demonstrating, conceivably, the presence of intact $^{32}\text{P}$-ODN1 at 2 min after onset of cell uptake. The amount of internalized $^{32}\text{P}$-ODN1 increased, reaching a maximum at 10–20 min of uptake.

In conclusion, liver KC and EC possess specific binding sites for ODNs. The inhibition profiles of the $^{32}\text{P}$-ODN1 binding site on both cell types are essentially equal and correspond closely to that of $^{125}\text{I}$-AcLDL binding receptors. Multiple scavenger receptors on nonparenchymal liver cells and bone marrow cells may participate in the elimination of phosphodiester ODNs from the bloodstream. These receptors are involved in the clearance of small DNA fragments from the circulation and supplement the DNA receptor described by Emlen et al. (1988), which is implicated in the removal and degradation of large and double-stranded DNA fragments.

Minor changes in ODN sequence markedly affect ODN recognition by these scavenger receptors and thus its in vivo fate; this further emphasizes that not only the intrinsic in vitro activity of an antisense ODN but also the biodistribution profile are crucial determinants of in vivo activity of a specific ODN. Through careful design of the antisense sequence, both nonparenchymal liver cell and bone marrow uptake can be modulated.

References


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