0026-895X/98/020262-08$3.00/0

MOLECULAR PHARMACOLOGY, **53:**262–269 (1998).

Liver Uptake of Phosphodiester Oligodeoxynucleotides Is Mediated by Scavenger Receptors

ERIK A. L. BIESSEN, HELENE VIETSCH, JOHAN KUIPER, MARTIN K. BIJSTERBOSCH, and THEO J. C. VAN BERKEL

Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, 2300 RA Leiden, The Netherlands

Received August 20, 1997; Accepted November 5, 1997

This paper is available online at http://www.molpharm.org

ABSTRACT

The therapeutic activity of antisense oligodeoxynucleotides (ODNs) often is impaired due to premature degradation and poor ability to reach the (intra)cellular 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, ^{32}P-ODNs (miscellaneous sequences and GT-containing ODNs with variable G content) are rapidly cleared from the bloodstream ($t_{1/2}$ = 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 ^{32}P-ODN uptake. To elucidate the mechanism of liver uptake, ^{32}P-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 ^{32}P-ODN binding to EC and KC by various (poly)anions were essentially equal and corresponded closely to those of ^{125}I-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; Rifai *et al.*, 1996; Sawai *et al.*, 1996). The qualitative studies of Emlen *et al.* (1988) and Rifai *et 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-I/II (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 leukocytes 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 ^{32}P-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 ^{32}P-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

This work was supported by Grant M93.001 from the Dutch Heart Foundation.

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 ODN 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), trypan blue, BSA (fraction V), levamisole·HCl, chloroquine diphosphate, polyadenosinic acid (potassium salt), polyguanosinic acid (potassium salt), polycytidinic acid (potassium salt), polyinosinic acid (potassium salt), heparin (from porcine intestinal mucosa, grade I), and fucoidin (from *Fucus vesiculosus*) were purchased from Sigma Chemical (St. Louis, MO). Salmon sperm DNA (native; molecular mass, 3.8×10^6 Da) and T4-polynucleotide kinase were purchased from Pharmacia (Woerden, The Netherlands). Monensin (sodium salt) was obtained from Calbiochem-Behring (La Jolla, CA). ODN were synthesized at the Department of Organic Chemistry (Dr. J. H. Van Boom) (5′-GAC.TT-T.AGT.CGT.CGT.GGA-cap, ODN1; 5′-TCC.ACG.ACG.ACT.AAA .GTC.TTT-cap, ODN2) or purchased from Eurogentec (Seraign, Belgium) (GTG.CCG.GGG.TCT.TCG.GGC-cap, ODN3; TTT.GTT.TGT. TTG.TTT.T, dG_3T_{13}; TGG.TGG.TGG.TGG.TGG.T, $dG_{10}T_6$; GGG.T-GG.GTG.GGT. GGG.G, $dG_{13}T_3$; GGG.GGG.GGG.GGG.GGG.G, dG_{16}).

***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 NaP_i, 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 $16{,}000 \times g$, 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 porta was cannulated, and the liver was perfused with Ca^{2+}-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 for determination of the total liver uptake. Subsequently, the liver was perfused with 0.01% (w/v) collagenase at 8° in Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4, and parenchymal and nonparenchymal cells were isolated as described previously (Van Berkel *et al.*, 1985). The nonparenchymal cell preparation was fractionated further into EC and KC by centrifugal elutriation as described in detail previously (Kuiper *et al.*, 1994). 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 the protein content, with the assumption that 92.5%, 2.5%, and 3.3% of the total liver protein content can be ascribed to the parenchymal cells, KC, and EC, respectively (Kuiper *et al.*, 1994). As found previously with other substrates (Van Berkel *et al.*, 1985; Kuiper *et al.*, 1994), 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, with the average value being 98 ± 5%. The purity of EC and KC preparations as monitored by peroxidase staining was ≥95% and ≥90%, respectively. The viability of the cells used for *in vitro* experiment was ≥90% as judged by 0.2% Trypan blue exclusion.

Isolation, modification, and radioiodination of human LDL. Human LDL ($1.024 < d < 1.055$) was isolated from human serum by two repetitive centrifugations according to Redgrave *et al.* (1975). After isolation, the purity and integrity of LDL were checked using agarose gel electrophoresis (0.8%) and PAGE. LDL was acetylated as described by Van Berkel *et al.* (1991). After modification, AcLDL was stored in PBS containing 2 mM EDTA at 4° under nitrogen and used within 4 weeks. The electrophoretic mobility of AcLDL on native agarose gel electrophoresis was 0.54 compared with 0.21 for native LDL. AcLDL was radioiodinated with $Na^{125}I$ to a specific activity of 110–230 cpm/ng as described previously (Van Berkel *et al.*, 1991).

Radiophosphorylation of ODNs. The ODNs were 5′-end labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase and monitored on purity and by gel electrophoresis on 19% polyacrylamide gel (80 mM Tris, 2 mM EDTA, 90 mM boric acid, 7 M urea, pH 8.8) (Sambrook *et al.*, 1989). Typically, the specific activity of ^{32}P-ODN was 100–250 cpm/ng.

***In vitro* binding of ^{32}P-ODN1 and ^{125}I-AcLDL to EC and KC.** Binding studies of ^{32}P-ODN1 to EC and KC were performed as described previously (Biessen *et al.*, 1994). KC (1.5×10^6; 150 μg of cell protein) or EC (2×10^6; 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.

For competition studies, KC and EC were incubated with ^{32}P-ODN1 (20 nM) or ^{125}I-AcLDL (5 μg/ml, 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 follows. KC (1.5×10^6; 150 μg of cell protein) or EC (2×10^6;150 μg of cell protein) in DMEM (0.5 ml) containing 2% (w/v) BSA were incubated for 10 min at 37° with radiolabeled ligand in the absence or presence of polyinosinic acid (100 μg/ml) or GMP (10 mM), 4-nitrophenylphosphate (10 mM), L-(+)-tartaric acid (1 mM), and levamisole (10 μM). After incubation, cells were placed on ice, washed with ice-cold DMEM plus 2.0% (w/v) BSA, and incubated for 10 min at 4° with DMEM plus 2.0% BSA plus polyinosinic acid [(w/v) 100 μg/ml] to remove membrane-associated ^{32}P-ODN1. In this time span, membrane-bound ^{32}P-ODN1 was almost quantitatively released from EC and KC (>90%). Subsequently, cells were processed as described before, and the cell-associated radioactivity was counted. Nonspecific uptake was defined as the binding of ^{32}P-ODN1 in the presence of 100 μg/ml polyinosinic acid. To test the effect of various agents that affect lysosomal uptake, cells were incubated for 30 min at 37° with DMEM plus 2.0% BSA in the absence or presence of NaN_3 (10 mM), chloroquin (1 mM), monensin (25 μM), or sucrose (250 mM). Subsequently, ^{32}P-ODN1 was added, and the cells were incubated and processed as described above.

Determination of protein, cholesterol, and carbohydrate content. Protein concentrations in cell suspensions were determined according to the method of Lowry *et al.* (1951), with BSA as the standard.

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 (Biessen *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_d = 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

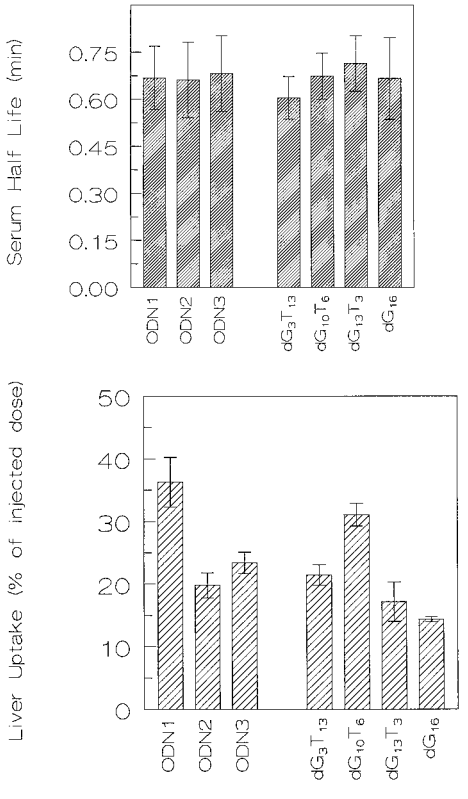

Fig. 1. Liver uptake (*top*) and serum half-life (*bottom*) of various ^{32}P-ODNs in the rat. ^{32}P-ODNs (ODN1, ODN2, ODN3, dG_3T_{10}, $dG_{10}T_6$, and dG_{16}; 4 μg) were injected intravenously into rats. At 1, 2, 5, 10, 20, 30, and 40 min after injection, radioactivities in serum was determined, 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_3T_{10}, $dG_{10}T_6$, and dG_{16}).

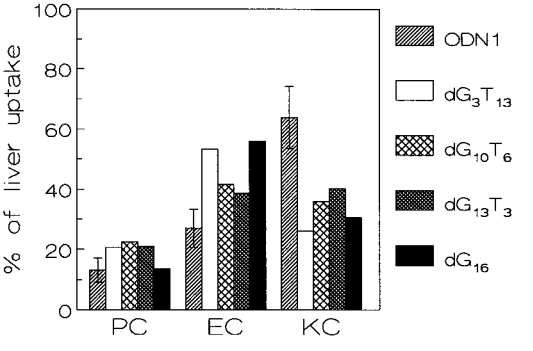

Fig. 2. Contribution of various cell types to the liver association of ^{32}P-ODN1. ^{32}P-labeled ODN (ODN1, dG_3T_{13}, $dG_{10}T_6$, $dG_{13}T_3$, and dG_{16}; 10 μg in 500 μl of PBS) was injected into rats. At 10 min after injection, parenchymal cells (*PC*), EC, and KC were isolated from the liver, 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.

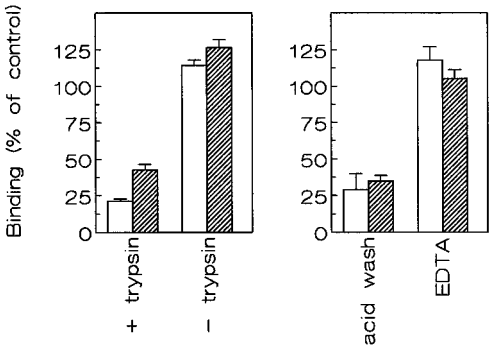

Fig. 3. *Left,* ^{32}P-ODN1 binding to EC (□) and KC (▨). Effect of trypsin digestion. EC or KC (2–3 × 10^6 cells) were incubated for 15 min at 37° in 1 ml of DMEM or trypsin (500 μg/ml) and subsequently washed thoroughly with DMEM supplemented with 2% BSA. Then, cells were incubated for 2 hr at 4° with ^{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 10.0 ± 0.9 ng/mg for EC and 4.5 ± 0.4 ng/mg for KC). *Right,* Effect of pH 2.8 shock or EDTA treatment. EC or KC (2–3 × 10^6 cells/ml) in 1 ml of DMEM were incubated for 2 hr at 4° with 20 nM ^{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° and subsequently washed. Cell-bound radioactivity was determined and corrected for protein content.

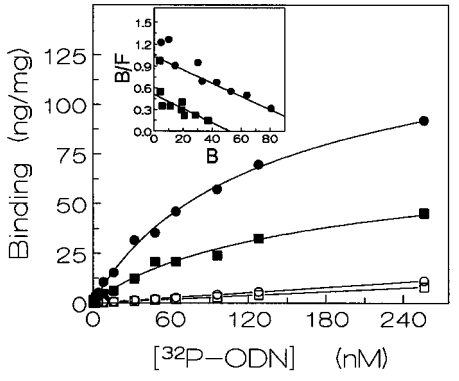

Fig. 4. Saturation curve of ^{32}P-ODN1 binding to EC and KC at 4°. EC (○, ●) or KC (□, ■) (2–3 × 10^6 cells/ml) were incubated for 2 hr at 4° with 0–250 nM ^{32}P-ODN1 in the absence (●, ■) or presence (○, □) of 100 μg of polyinosinic acid. After incubation, the cells were washed thoroughly, and the 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.

types. ^{32}P-ODN1 binding to nonparenchymal liver cells was not influenced by incubation with EDTA (10 mM), indicating that ^{32}P-ODN1 binding is a Ca^{2+}-independent process.

Subsequently, we investigated the effect of various anionic competitors on the binding of ^{32}P-ODN1 to EC and KC (Fig. 5). ^{32}P-ODN1 binding was inhibited for 85–90% by polyinosinic acid (100 μg/ml) and polyguanosinic acid (100 μg/ml), whereas polyadenosinic acid (100 μg/ml) and polycytidinic acid (100 μg/ml) were not effective. In addition, it was demonstrated that the ability of mononucleotides to inhibit ^{32}P-ODN1 binding decreased from ATP and GTP (data not shown) via ADP to AMP, which was only a poor inhibitor at 50 mM. Similarly, the potency of phosphodiester 16-mers to interfere with ^{32}P-ODN1 binding increased with increasing G content from an insignificant 5% inhibition for dG_3T_{13} (at 1 μM) to 40% for $dG_{10}T_6$ and even 90% inhibition for dG_{16}. Of the other polyanions, only fucoidin gave full inhibition of

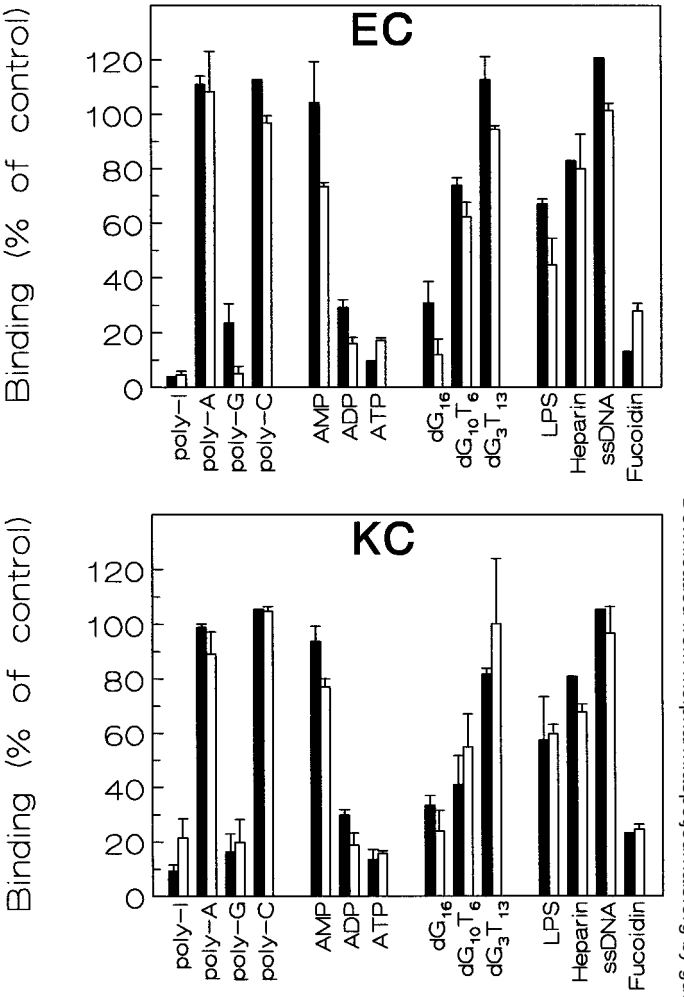

Fig. 5. Effect of various (poly)anions on the binding of ^{32}P-ODN1 (*closed bars*) or ^{125}I-AcLDL (*open bars*) to EC (*top*) and KC (*bottom*). EC or KC (2–3 × 10^6 cells/ml) were incubated in DMEM (supplemented with 2% BSA), with ^{32}P-ODN1 (20 nM) or ^{125}I-AcLDL (9.6 nM), in the absence (control) or presence of polyinosinic acid (*polyI*, 100 μg/ml), polyadenosinic acid (*polyA*, 100 μg/ml), polyguadinilic acid (*polyG*, 100 μg/ml), polycytidinic acid (*polyC*, 100 μg/ml), AMP (50 mM), ADP (50 mM), ATP (50 mM), dG_{16} (1 μM), $dG_{10}T_6$ (1 μM), dG_3T_{13} (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°. 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–

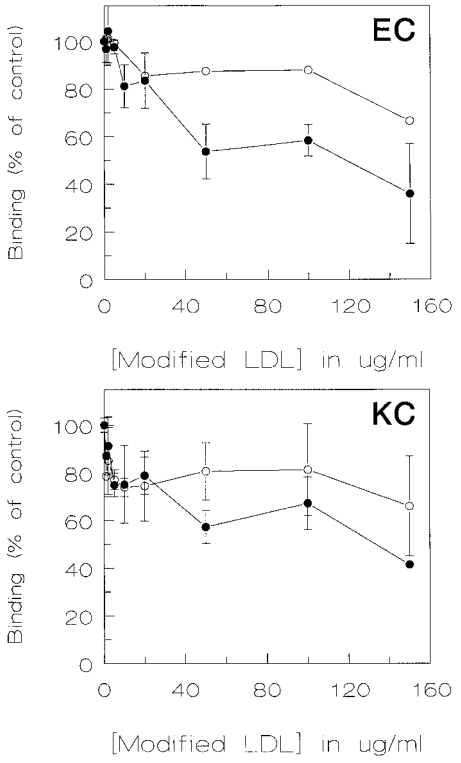

Fig. 6. Effect of AcLDL (●) and OxLDL (○) on ^{32}P-ODN1 binding to EC (*top*) and KC (*bottom*). EC or KC (2–3 × 10^6 cells/ml in DMEM plus 2% BSA) were incubated for 2 hr at 4° with 20 nM ^{32}P-ODN1 and 0–150 μg/ml AcLDL or OxLDL. After incubation, the cells were washed thoroughly, and the cell-bound radioactivity was determined. ^{32}P-ODN1 binding is plotted as percentage of total binding (in the absence of displacer) and is the mean ± standard deviation of six experiments.

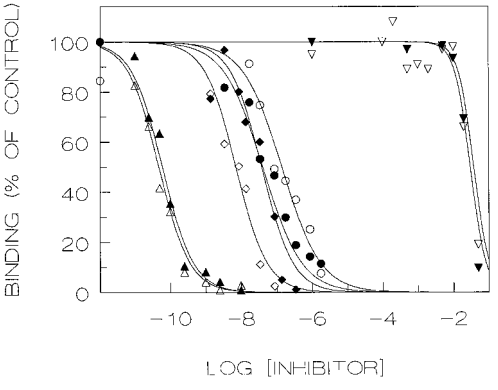

Fig. 7. Competition curves of ^{32}P-ODN1 binding to EC (*closed symbols*) and KC (*open symbols*). Liver EC and KC were incubated for 2 hr at 4° with 20 nM ^{32}P-ODN1 and polyinosinic acid (▲, △), unlabeled ODN1 (●, ○), ODN3 (◇, ◆), or ADP (▽, ▼). The 100% specific binding is defined as the difference of ^{32}P-ODN1 binding in the presence and absence of 100 μg/ml polyinosinic acid and is determined in duplicate.

20% inhibition; however, there was a considerable interexperimental variation in the effect of both modified lipoproteins on ^{32}P-ODN binding.

The efficacy of two ODNs (ODN1 and ODN3), the polyanion polyinosinic acid, and the mononucleotide ADP to displace ^{32}P-ODN1 binding to EC and KC was studied in more detail using competition binding studies (Fig. 7). All compounds fully inhibited specific ^{32}P-ODN1 binding in a competitive fashion with inhibition constants of 74 ± 23 nM (unlabeled ODN1), 10 ± 3 nM (ODN3), 0.052 ± 0.008 nM

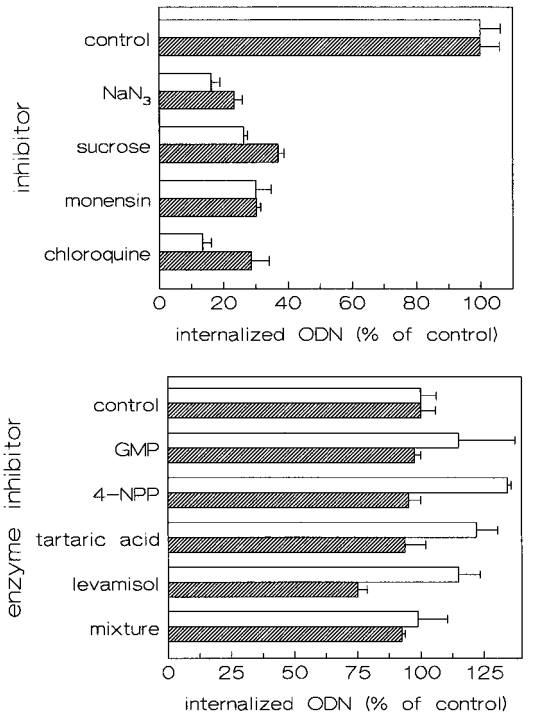

Fig. 8. *Top*, effect of classic uptake inhibitors on the rate of initial ^{32}P-ODN1 uptake by EC (□) and KC (▨). EC or KC (2–3 × 10^6 cells/ml) were incubated for 30 min at 37° with sodium azide (NaN_3, 10 mM), sucrose (250 mM), monensin (10 μM), colchicine (100 μM), or chloroquine (1 mM). Subsequently, the cells were incubated for 10 min at 37° with 20 nM ^{32}P-ODN1 in DMEM plus 2% BSA. After incubation, the cells were put on ice, washed once with DMEM plus 2% BSA, and incubated with polyinosinic acid (100 μg/ml in DMEM plus 2% BSA) to remove membrane-associated radioactivity. Finally, the cells were washed thoroughly, and the cell-associated radioactivity was determined. Uptake is given as percentage of initial uptake in the absence of displacer. *Bottom*, effect of enzyme inhibitors on the rate of initial ^{32}P-ODN1 uptake by EC (□) and KC (▨). EC or KC (2–3 × 10^6 cells/ml) were incubated in DMEM (supplemented with 2% BSA) for 10 min at 37° with 20 nM ^{32}P-ODN1 in the absence or presence of GMP (10 mM), 4 nitrophenol phosphate (*4-NPP*, 10 mM), tartaric acid (1 mM), levamisol (10 μM), or a mixture of GMP, 4-NPP, tartaric acid, and levamisol (*mix*). After incubation, the cells were put on ice, washed once with DMEM plus 2% BSA, and incubated with polyinosinic acid (100 μg/ml in DMEM plus 2% BSA) to remove membrane-associated radioactivity. Finally, the cells were washed thoroughly, and the cell-associated radioactivity was determined. Uptake is given as percentage of initial uptake in the absence of displacer.

(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 ^{32}P-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 ^{32}P-ODN1 by treatment with 100 μg/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 ^{32}P-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 ^{32}P-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, ^{32}P-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; levamisole, a alkaline phosphatase inhibitor; and a mixture of these agents had no affect 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° 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°. Degradation products of ^{32}P-ODN1 were not observed during this time course.

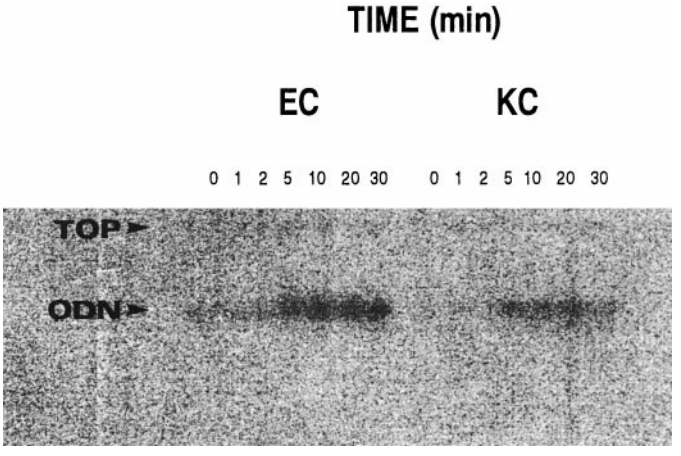

Fig. 9. PAGE analysis of the kinetics of ^{32}P-ODN1 internalization by EC and KC. EC or KC (2–3 × 10^6 cells/ml) were incubated in DMEM (supplemented with 2% BSA) with 20 nM ^{32}P-ODN1 for 0, 1, 2, 5 10, 20, or 30 min at 37°. After incubation, the cells were put on ice, washed once with ice-cold DMEM (1 ml; supplemented with 2% BSA), and incubated for 5 min at 4° with polyinosinic acid (100 μg/ml in DMEM plus 2% BSA) to remove membrane-bound ODN1. Subsequently, the cells were washed thoroughly, heated for 5 min at 96°, 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 M ureum), and the gel was autoradiographed using the PhosphorImager.

Discussion

A number of studies have shown that phosphodiester ODNs injected intravenously into rats are eliminated from the circulation partly as a result of rapid redistribution over the body fluid and partly as a result of renal excretion and extensive liver uptake (Emlen *et al.*, 1988; De Smidt *et al.*, 1991 ; Zendegui *et al.*, 1992; Inagaki *et al.*, 1994; Sands *et al.*, 1994; Rifai *et al.*, 1996; Sawai *et al.*, 1996). A quantitative analysis of the cell types responsible for this liver uptake and a study of the actual uptake mechanism are still lacking. This prompted us to analyze the intrahepatic cellular distribution of ^{32}P-ODNs and characterize the recognition site or sites responsible for this uptake. In agreement with previous studies (De Smidt *et al.*, 1991; Zendegui *et al.*, 1992; Inagaki *et al.*, 1994; Sands *et al.*, 1994; Rifai *et al.*, 1996), all of the tested phosphodiester ODNs were cleared rapidly from the bloodstream. Liver uptake of the miscellaneous ODNs ranged from 22% to 36%, whereas for GT-containing model ODNs, uptake ranged from 15% to 31%. Liver uptake was maximal for ODNs with an intermediate G content and markedly reduced for G-rich ODNs (>50–60%). The reduced uptake was compensated for by an enhanced bone marrow disposition, possibly by the dextran-sensitive binding site for ODNs on pro-B/pre-B cells (Zhao *et al.*, 1994).

Tissue distributions of the ^{32}P-ODNs were similar and indicative of redistribution of the ODNs and/or their metabolites over the total body fluid. Apart from the nonparenchymal liver cells, specific disposition of radioactive ODNs was observed only in bone marrow (for G-rich GT-containing ODNs) and in the intestine, the latter probably reflecting hepatic degradation of ^{32}P-ODNs and subsequent biliary secretion. Hepatic uptake of ODNs could be ascribed mainly to nonparenchymal liver cells, which concurs with the qualitative findings of Emlen *et al.* (1988), Rifai *et al.* (1996), and Inagaki *et al.* (1994) that, in rats, both DNA and phosphorothioates are taken up rapidly by adherent cells in the liver. No correlation was found between ODN sequence and the ratio of KC to EC uptake.

These results are surprising in view of the fact that G-rich ODNs display a higher affinity for scavenger receptor class A-I/II (Pearson *et al.*, 1993), which is localized mainly on liver EC (Van Berkel *et al.*, 1991: Nagelkerke *et al.*, 1983). It therefore was anticipated that G-rich ODNs would be eliminated more efficiently from the bloodstream by hepatic EC. Additional receptor systems (Suzuki *et al.*, 1997) on EC, KC, and bone marrow cells, with similar substrate recognition profiles, also probably contribute to ODN clearance.

The mechanism of the rapid and substantial hepatic uptake of ODNs *in vivo* was addressed further *in vitro* by studying ^{32}P-ODN1 binding to isolated EC and KC. ^{32}P-ODN1 binding to both cell types seemed to be saturable and of moderately high affinity, involves a protein receptor, and is Ca^{2+} independent. The latter makes it unlikely that the ^{32}P-ODN1 binding site is identical to the DNA receptor on leukocytes, which was reported to be Ca^{2+} dependent (Bennett *et al.*, 1985). Likewise, the recently identified ODN receptor MAC-1 (CD11b/CD18; Benimetskaya *et al.*, 1997) differs from the hepatic ^{32}P-ODN binding site on EC and KC in that ODN binding to MAC-1 is Ca^{2+} dependent.

To characterize the ^{32}P-ODN1 recognition site or sites on nonparenchymal liver cells in more detail, the binding profile

was monitored using displacement studies. Of the tested polyanions, only unlabeled ODN1, polyinosinic acid, polyguanosinic acid, and fucoidin inhibited ^{32}P-ODN1 binding, whereas structural analogues like polyadenosinic and polycytidinic acid were not capable of displacing ^{32}P-ODN1 binding. In concert with the data of Pearson *et al.* (1993), the ability of 16-mers to displace ^{32}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}P-ODN1 recognition sites on both cell types are identical. Second, the binding characteristics of ^{32}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}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}P-ODN1 binding to nonparenchymal liver cells. Third, the characteristics of ^{32}P-ODN1 binding to EC and KC closely parallel that of ^{125}I-AcLDL binding to these cell types. Recent studies have shown that a number of scavenger receptors are able to take up ^{125}I-AcLDL, including scavenger receptor class A-I/II (Horiuchi *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 Fcγ/RII 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 vitro* 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}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}I-AcLDL binding to both cell types (271 $\pm$ 33 and 58 $\pm$ 19 fmol/mg of cell protein, respectively; three experiments; data not shown).

Uptake studies at 37° demonstrated that ^{32}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}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 (sucrose), 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 ($\pm$90%) and, to a lesser extent, uptake ($\pm$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, and phosphatase inhibitors nor a mixture of these agents significantly affected ODN uptake, suggesting that intact ^{32}P-ODN1 is internalized. This is confirmed by autoradiographic analysis of EC and KC cell lysates, demonstrating, inconceivably, the presence of intact ^{32}P-ODN1 at 2 min after onset of cell uptake. The amount of internalized ^{32}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}P-ODN1 binding site on both cell types are essentially equal and correspond closely to that of ^{125}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 denominators 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

Ashkenas J, Penman M, Vasile E, Acton S, Freeman M, and Krieger M (1993) Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. *J Lipid Res* **34:**983–1000.

Benimetskaya L, Loike JD, Khaled Z, Loike G, Silverstein SC, Cao L, El Khoury J, Cai TQ, and Stein CA (1997) Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. *Nat Med* **3:**414–420.

Bennet RM, Gabor GT, and Merritt MM (1985) DNA binding to leukocytes. *J Clin Invest* **76**:2182–2190.

Biessen EAL, Bakkeren HF, Beuting DM, Kuiper J, and Van Berkel ThJC (1994) Recognition of both fucose- and galactose-exposing particles by the hepatic fucose receptor depends on the particle size. *Biochem J* **299:**291–296.

Biessen EAL, Norder JA, Horn AS, and Robillard GT (1988) Evidence for the existence of at least two different binding sites for 5-HT-reuptake inhibitors within the 5-HT-reuptake system from human blood platelets. *Biochem Pharm* **37:**3959–3966.

Bijsterbosch MK, Ziere GJ, and Van Berkel ThJC (1989) Lactosylated low-density lipoprotein: a potential carrier for the site-specific delivery of drugs to Kupffer cells. *Mol Pharmacol* **36:**484–489.

Caster WO, Simon AB, and Armstrong WD (1955) Evans blue space in tissues of the rat. *Am J Physiol* **183:**317–321.

Crooke ST and Bennet CF (1996) Progress in antisense oligonucleotide therapeutics. *Annu Rev Pharmacol* **36:**107–129.

De Rijke YB, Biessen EAL, Vogelezang CJM, and Van Berkel ThJC (1994) Binding characteristics of scavenger receptors on liver endothelial and Kupffer cells for modified low-density lipoproteins. *Biochem J* **304:**69–73.

De Smidt PC, Le Doan T, De Falko S, and Van Berkel ThJC (1991) Association of antisense oligonucleotides with lipoproteins prolongs the plasma half-life and modifies the tissue distribution. *Nucleic Acid Res* **19:**4695–4700.

Doi T, Higashino K, Kurihara Y, Wada Y, Miyazaki T, Nakamura H, Uesugi S, Imanishi T, Kawabe Y, Itakura H, Yazaki Y, Matsumoto A, and Kodama T (1993) Charged collagen structure mediates the recognition of negatively charged macromolecules by macrophage scavenger receptors. *J Biol Chem* **268:**2126–2133.

Emlen W, Rifai A, Magilavy D, and Mannik M (1988) Hepatic binding of DNA is mediated by a receptor on nonparenchymal cells. *Am J Pathol* **133:**54–60.

Endeman G, Stanton LW, Madden KS, Bryant CM, White RT, and Protter AA (1993) CD36 is a receptors for oxidized low density lipoprotein. *J Biol Chem* **268**:11811–11816.

Hampton RY, Golenbak DT, Penman M, Krieger M, and Raetz CR (1991) Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature (Lond)* **353:**342–344.

Horiuchi S, Takata K, Maeda H, and Morino Y (1985) Scavenger receptor function of sinusoidal liver cells: acetylated low-density-lipoprotein is endocytosed via a route distinct from formaldehyde-treated serum albumin. *J Biol Chem* **259:**53–56.
Inagaki M, Togawa K, Carr BI, Ghosh K, and Cohen JS (1994) Antisense oligonucleotides: inhibition of liver cell proliferation and in vivo disposition. *Transplant Proc* **24:**2971–2972.
Kuiper J, Bakkeren HF, Biessen EAL, and Van Berkel ThJC (1994) Characterisation of the interaction of galactose-exposing particles with rat Kupffer cells. *Biochem J* **299:**285–290.
Loke S, Stein C, and Zhang X (1989) Characterization of oligonucleotide transport into living cells. *Proc Natl Acad Sci USA* **86:**3474–3478.
Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.
Nagelkerke JF, Barto KP, and Van Berkel ThJC (1983) In vivo and in vitro uptake and degradation of acetylated LDL by rat liver endothelial, Kupffer and parenchymal cells. *J Biol Chem* **258:**12221–12227.
Pearson AM, Rich A, and Krieger M (1993) Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. *J Biol Chem* **268:**3546–3554.
Redgrave TG, Roberts DCK, and West CE (1975) Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal Biochem* **65**:42–49.
Rifai A, Brysch W, Fadden K, Clark J, and Slingensiepen KH (1996) Clearance kinetics, biodistribution, and organ saturability of phosphorothioate oligodeoxynucleotides in mice. *Am J Pathol* **149:**717–725.
Sambrook J, Frits EF, and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual,* 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sands H, Gorey-Feret LJ, Cocazza AJ, Hobbs FW, Chidester D, and Trainer GL (1994) Biodistribution and metabolism of internally ^{3}H-labeled oligonucleotides. *Mol Pharmacol* **45:**932–943.
Sawai K, Mahato RI, Oka Y, Takakura Y, and Hashida M (1996) Disposition of oligonucleotides in isolated perfused rat kidney: involvement of scavenger receptors in their renal uptake. *J Pharmacol Exp Ther* **279:**284–290.
Stanton LW, White RT, Bryant CM, Protter AA, and Endemann G (1992) A macrophage Fc receptor for IgG is also a receptor for oxidized low density lipoproteins. *J Biol Chem* **267:**22446–22451.
Stein CA and Cheng YC (1993) Antisense oligonucleotides as therapeutic agents: is the bullet really magical? *Science (Washington DC)* **261:**1004–1011.
Stein CA, Tonkinson JL, Zhang LM, Yabukov L, Gervasoni J, Taub R, and Rotenberg SA (1993) Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells. *Biochemistry* **32:**4855–4861.
Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, Takashima Y, Kawabe Y, Cynshi O, Wada Y, Honda M, Kurihara H, Aburatani H, Doi T, Matsumoto A, Azuma S, Noda T, Toyoda Y, Itakura H, Yazaki Y, Horiuchi K, Takahashi K, Kruijt JK, Van Berkel ThJC, Steinbrecher UP, Ishibashi S, Maeda N, Gordon S, and Kodama T (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature (Lond)* **386:**292–296.
Van Berkel ThJC, De Rijke YB, and Kruijt JK (1991) Different fate in vivo of oxidatively modified low-density lipoprotein in rats. *J Biol Chem* **266:**2282–2289.
Van Berkel ThJC, Kruijt JK, Spanjer HH, Nagelkerke JF, Harkes L, and Kempen HJM (1985) The effect of a water-soluble Tris-galactoside terminated cholesterol derivative on the fate of low-density lipoproteins and liposomes. *J Biol Chem* **260:**2694–2699.
Van Berkel ThJC, Nagelkerke JF, and Kruijt JK (1981) The effect of Ca^{2+} and trifluoperazine on the processing of human acetylated low-density-lipoprotein by non-parenchymal liver cells. *FEBS Lett* **132:**61–66.
Yabukov LA, Deeva EA, Zarytova VF, Ivanova EM, Ryte AS, Yurchenko LV, and Vlassov VV (1989) Mechanism of oligonucleotide uptake by cells: involvement of specific receptors. *Proc Natl Acad Sci USA* **86:**6454–6458.
Zendegui JG, Vasquez KM, Tinsley JH, Kessler DJ, and Hogan ME (1992) In vivo stability and kinetics of absorption and disposition of 3′ phosphopropyl amine oligonucleotides. *Nucleic Acids Res* **20:**307–314.
Zhao Q, Matson S, Herrera CJ, Fisher E, Yu H, and Krieg AM (1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. *Antisense Res Dev* **3:**161–171.
Zhao Q, Waldschmidt T, Fisher E, Herrera CJ, and Krieg AM (1994) Stage-specific oligonucleotide uptake in murine bone marrow B-cell precursors. *Blood* **84:**3660–3666.

Send reprint requests to: Dr. E. A. L. Biessen, Division of Biopharmaceutics, Sylvius Laboratories, University of Leiden, P.O. Box 9503, 2300 RA Leiden, The Netherlands.