Antisense DNA Delivery In Vivo: Liver Targeting by Receptor-Mediated Uptake

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Antisense oligodeoxynucleotides coupled to asialoglycoprotein carrier molecules were evaluated in terms of their ability to accumulate preferentially in the liver and thus potentially serve as an important method to regulate liver gene expression. **Methods:** Native and asialo-human alpha-1 acid glycoproteins were derivatized with low molecular weight poly(L)lysine and complexed with an antisense DNA (67 mer) complementary to the 5′ end of rat serum albumin mRNA. The asialoglycoprotein antisense complex (conjugate) was characterized with respect to size, stability, and anti-sense loading, and the biodistribution of the conjugate was determined for normal rats at 5 min and 1, 6, and 24 hr after intravenous injection. In vivo stability of the anti-sense asialoglycoprotein complex was also evaluated using double-labeled (^{32}P-antisense and ^{3}H-glycoprotein) preparations. **Results:** The results of the conjugate characterization studies demonstrated that at least 30% of the anti-sense DNA dissociated from the carrier after 7 min under chromatographic conditions. When the conjugate was incubated with PBS, MEM or MEM plus 10% FBS for 1 hr at 37°C, about 85% of the antisense DNA was dissociated from the carrier. The results of the biodistribution studies showed that the accumulation of the asialo-glycoprotein anti-sense complex in the liver was rapid and greatly exceeded the accumulation of the sialo-glycoprotein antisense analog or antisense alone. **Conclusion:** These findings have significant implications for the targeted delivery of therapeutic antisense molecules to the liver.

Key Words: antisense DNA delivery; receptor-mediated uptake; asialoglycoprotein; oligodeoxynucleotides; sialoglycoprotein

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Antisense oligodeoxynucleotides have been widely used for the regulation of gene expression in cell culture, plant engineering, and the design of chemotherapeutic agents (*1*). In principle, an antisense oligonucleotide can be used to target any known sense RNA at the CAP or AUG regions, the beginning of the poly A chain, or even some segments of RNA. Antisense reagents are believed to block the expression of targeted proteins by hybridizing with their mRNA and preventing its translation.

In order to achieve adequate intracellular concentrations of antisense reagents, it is often necessary to administer the antisense at or close to cytotoxic concentrations. One approach to increase intracellular antisense concentration and achieve selective delivery to specific cell types is to use biological ligands as carrier molecules. These ligands will selectively bind to their specific receptor and be internalized by the cell via receptor-mediated endocytosis. Two approaches have been employed for the targeted delivery of nucleic acids with biological ligand molecules. The first method reported by Wu et al. (*2*) takes advantage of a noncovalent salt bridge formation between a positively charged poly(L)lysine carrier and negatively charged plasmid vector. Using an asialoglycoprotein moiety as the biological ligand, the conjugate was efficiently targeted to the asialo-glycoprotein receptor found on hepatocytes. The conjugate was internalized and proteins encoded by the plasmid vector were detected in both in vitro and in vivo model systems.

In the second approach, liposomes were prepared from a solution containing a high concentration of antisense oligonucleotide. In one example, the liposome carrier was further modified by the addition of protein A, which binds to the Fc segment of antibodies on target cells (*3*). This technique has been used successfully in vitro, but in vivo applications have not been reported.

Although antisense reagents have been used for both in vitro and in vivo targeting, the factors contributing to effective delivery in vivo have not been evaluated in detail. In the present study, we document some of the critical parameters for efficient receptor-mediated delivery of antisense to the liver in vivo. The therapeutic implications of this approach to the treatment of liver disease are discussed.

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MATERIALS AND METHODS

Reagents and Solutions

Glycoprotein (human alpha-1 acid), poly(L)lysine (MW 3,000), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), sodium metaperiodate, sodium acetate, sodium sulfate, sodium arsenite phosphotungstic acid, thiobarbituric acid, a protein assay kit (P-5656) and polynucleotide kinase (P-4390) were purchased from Sigma Chemical Company (St. Louis, MO). Neuraminidase (EC 3.2.1.18) from clostridium perfringens and mucin were obtained from Worthington Biochemical Corporation (Freehold, NJ). The fluorescent label, FTIC, was purchased from Molecular Probes (Eugene, OR). [Gamma-^{32}P]-adenosine 5'-triphosphate tetra-(triethylammonium)salt (222TBq/mmole, NEG002Z) and N-succinidyl [2,3-^{3}H] propionate (1757.5 GBq/mmole, NET 632), Solvable (NEF-910) and Formula 989 (NEF-989) were purchased from Dupont New England Nuclear Corp. (Billerica, MA).

T4 kinase buffer (10 ×)$_2$ was prepared from: 700 m*M* Tris-HCl, pH = 7.6, 100 m*M* $MgCl_2$, 1 m*M* KCl and 50 m*M* dithiothreitol. Deionized water was produced with an ion exchange unit, Millipore (Milli-Q). Triethylamine acetate solution (0.5 *M*) was prepared by adding 50.6 g of triethylamine to 0.95 liter of Milli-Q water followed by titration to pH 6.6 with glacial acetic acid adjusting to a final volume of 1 liter with Milli-Q water. All buffer solutions and Milli-Q water were sterilized by filtration through a 0.22 μM membrane.

Preparation of Antisense DNA

An antisense DNA oligonucleotide (67 mer), which is complementary to 5' end of the mRNA encoding rat serum albumin, was synthesized with an Applied Biosystems (Foster City, CA) oligonucleotide synthesizer. After deprotection, the oligomer was dissolved in water, extracted with phenol/chloroform (1:1, vol/vol), precipitated with ethanol and redissolved in sterile water. The purity of the product was evaluated by measuring the absorbance ratio at 260 nm to 280 nm and reversed-phase HPLC (Column—Aquapore RP-300, 250 × 4.6 mm i.d.; mobile phase A—0.5 *M* triethylamine acetate; mobile phase B—100% ACN; linear gradient—2%–30% B over 30 min: Detection—UV 260 nm).

Preparation of Asialoglycoprotein

Neuraminidase (10 units, ~0.25 mg protein) was dissolved in 250 μl of water (1 mg/ml). Human alpha-1 acid glycoprotein (128 mg) was dissolved in 4 ml of water, the pH was adjusted to 5.0 with 0.5 ml acetic acid (0.1 *M*, pH = 5.0) and the volume of the final solution was adjusted to 5 ml with Milli-Q water. Neuraminidase solution (100 μl) was added to the glycoprotein solution and the mixture was incubated at 37°C for 1 hr with gentle shaking. Measurement of sialic acid released from the glycoprotein was performed according to the manufacturer's instructions. The final solution was dialysed twice against PBS at 4°C (MW cut off 8000). The molecular weight of the asialoglycoprotein was determined with a TSK 2000SW column eluted with 0.1 *M* sodium phosphate/0.1 *M* sodium sulfate buffer, pH = 6.3 at a flow rate of 1 ml/min. The column was calibrated with the following standards: IgG (MW = 150,000), BSA (MW = 66,000), pepsin (MW = 35,000), cytochrome C (MW = 12,400) and poly(L)lysine (MW = 3,000). The detection wavelengths were 280 nm for protein and 220 nm for poly(L)lysine. A Beckman System Gold HPLC equipped with a dual wavelength detector (Beckman 167) was used for all chromatographic separations. The concentration of the stock asialoglycoprotein solution was determined by the modified Lowry method (*4*) and the molecular weight of the protein was measured with a precalibrated TSK 2000SW column. The molecular weight of the antisense oligonucleotide was calculated to be 21,200. The molar extinction coefficients of the asialoglycoprotein and the antisense oligonucleotide were determined at 280 and 260 nm, respectively.

Preparation of Poly(L)Lysine Modified Asialoglycoprotein

Asialoglycoprotein was modified with poly(L)lysine by the procedure reported by Wu et al. (*5*). Briefly, asialoglycoprotein (1 μmole) was treated with poly(L)lysine (7 μmole) in PBS (pH 7.4) containing excess EDAC (154 μmole) for 24 hr at 4°C. The asialoglycoprotein-poly(L)lysine-to-asialoglycoprotein ratio in the purified product was determined with a precalibrated TSK 2000SW column.

Preparation of Asialoglycoprotein-Poly(L)Lysine-FITC

FITC was covalently linked to poly(L)lysine by standard procedures. Briefly, poly(L)lysine (6.6 μmole) dissolved in phosphate buffer (0.1 *M*, pH = 8.0) was added to a stirred solution of FITC (13 μmole) and the mixture was incubated at room temperature for 3 hr. Covalent coupling of asialoglycoprotein (0.25 μmole) to poly(L)lysine-FITC (1.7 μmole) was performed with EDAC (0.25 μmole) as described above. The asialoglycoprotein-poly(L)lysine-FITC was purified on a Sephacryl S-300 column eluted with PBS. The purity of the final product was determined by HPLC and UV spectroscopy.

Confocal Microscopy of Rat Hepatocytes

Rat hepatocytes were isolated by a two-step liver perfusion protocol (*6*). An aliquot of the cell suspension (1.6 × 10^6 cell/ml, 2 ml) was transferred to a petri dish (60 mm) that was precoated with a 1-ml solution of collagen gel (type 1, isolated from rat tail tendon, 1.1 mg/ml). Cell attachment was achieved by incubation at 37°C for 2 hr. The medium was changed, and cells were washed with 2 ml of MEM containing 20 m*M* Hepes and 5 m*M* $CaCl_2$. Finally, 2 ml of pre-warmed MEM containing asialoglycoprotein-poly(L)lysine-FITC (1.2 mg/ml, 0.1 ml) was added to the dish and the mixture was incubated for 1 hr at 37°C in humidified 10% CO_2. The reaction was stopped by washing twice with pre-chilled MEM (without Ca^{2+}) at 4°C. The intracellular distribution of the asialoglycoprotein-poly(L)lysine-FITC was studied with a confocal microscope (MRC-600, Bio-Rad, Cambridge, MA).

Phosphorus-32 Labeling of Antisense DNA

Antisense DNA (OD_{260} = 18, 35 μl) was mixed on ice with spermidine (10 m*M*, 10 μl), 10 × 5'-kinase buffer (15 μl), gamma-^{32}P ATP (1 mCi, 100 μl) and T4 kinase (50 units, 5 μl) in a 1.5-ml reaction vial. After 1 hr of incubation in a water bath at 37°C, LiCl solution (4 *M*, 17 μl) was added with vigorous mixing. Ethanol (95%, 550 μl) was added and the antisense solution was chilled on dry ice for 30 min. The ^{32}P-labeled antisense DNA was pelleted at 12,000 × g for 20 min at 4°C, and the pellet was washed twice with 70% ethanol (1 ml), centrifuged, and re-suspended in sterile Milli-Q water (50 μl).

Tritium Labeling of Sialoglycoprotein

N-succinidyl [2,3-^{3}H] propionate (37 MBq) was dried under a gentle stream of N_2 in order to remove excess organic solvents. Sialoglycoprotein (7.3 mg/ml, 1.5 ml in 0.1 *M* phosphate buffer, pH 7.4) was added with stirring and the reaction mixture incubated at room temperature for 3 hr. The sialoglycoprotein was further modified with poly(L)lysine as described above. The final product was purified on a Sephacryl S-300 column eluted with

PBS, and concentrated by using a centriprep device (MW cutoff: 10,000) to 0.2 ml (OD_{280} = 2.7, ^3H radioactivity = 0.35 MBq, ~100% coupled).

Sialoglycoprotein-Poly(L)Lysine: Antisense Complex

Tritium-3-sialoglycoprotein-poly(L)lysine (OD_{280} = 2.7, 80.3 KBq, 400 μl) was added to the ^{32}P-labeled antisense (412 KBq) with vigorous mixing. The solution was incubated at 4°C overnight and dialysized (2×) against PBS (1500:1, membrane MW cutoff: 3500). The product was characterized by agarose gel electrophoresis and HPLC. Agarose gel (2%) electrophoresis was performed using the method reported by Wu et al. (*2*). For HPLC analysis, ^3H-sialoglycoprotein-poly(L)lysine: ^{32}P-antisense (20 μl) was applied to a precalibrated TSK 2000SW column eluted with 0.1 *M* sodium phosphate/0.1 *M* sodium sulfate, pH 6.5 at a flow rate of 1.0 ml/min and monitored at 280 nm. In addition, 1-ml fractions of the elution were collected in liquid scintillation vials. In this way, A_{280} absorbance data (total protein), ^3H (sialoglycoprotein-poly(L)lysine) and ^{32}P (antisense) chromatograms were simultaneously acquired.

Antisense and Protein Molecule Biodistribution

Twenty-four male Sprague-Dawley rats (mean weight = 124.87 g, s.d. = 10.14 g) were injected via the tail vein with ^3H-sialoglycoprotein-poly(L)lysine: ^{32}P-antisense complex (150 μl, 555 KBq —^{32}P, 122.0 KBq—^3H for each animal). At 5 min, 60 min, 6 hr and 24 hr after injection, a group of six rats were killed and samples (~100 mg) of blood, heart, lung, liver, spleen, kidney, adrenal, stomach, GI-tract, testes, brain and muscle were accurately weighed in glass liquid scintillation vials. Solvable (DuPont New England Nuclear Corp., Billerica, MA) (1 ml) was added to each vial and the samples were incubated at 50°C for 1 hr. Some tissues (such as spleen and lung) solubilized slowly and were ground to a cloudy suspension with a glass rod. All tissue samples were completely dissolved after an additional hour of incubation at 50°C. For the blood samples, EDTA (100 m*M*, 0.1 ml) was added to prevent foaming. All samples were bleached with 30% hydrogen peroxide (0.1 ml), and allowed to stand at room temperature for ~30 min. Ten milliliters of Formula 989 (DuPont New England Nuclear) were added to each vial, and the vials were shaken for a few seconds. Hydrochloric acid (0.5 N, 0.5 ml) was added to the blood samples. These samples were shaken and allowed to stand for 5 min.

Radioactivity measurements were performed with a liquid scintillation counter that had been precalibrated with ^3H and ^{32}P standards. All radioactivity measurements were performed for a preset time of 1 min.

Internal standards were prepared by adding aliquots of ^3H and ^{32}P to five vials followed by Formula 989 (10 ml). After performing the tissue radioactivity measurements, an aliquot of the internal standard was added to each sample and radioactivity was measured again. In this way, the efficiency of ^3H and ^{32}P detection was determined for all tissues.

A control biodistribution study was performed to evaluate the behavior in vivo of ^{32}P-antisense alone. Twenty-four male Sprague-Dawley rats (mean weight = 120.19 g, std. = 13.32 g) were injected with ^{32}P-antisense and radioactivity was measured as described above.

Finally, the biodistribution of the asialoglycoprotein poly(L) lysine antisense conjugate was studied. Twenty-four male Sprague-Dawley rats (mean weight = 116.18 g, std. = 8.72 g) were injected with asialoglycoprotein-poly(L)lysine: ^{32}P-antisense via the tail vein. The protocol was performed as described above.

Statistical Methods

The results of the biodistribution studies were evaluated by analysis of variance (ANOVA) with a linear model in which organ and time were the classification variables: %ID/g or %ID/organ = Organ + time + Organ * Time. Post-hoc comparison of peptide concentration was performed by Duncan's new multiple-range test (*7*). The first subscript of each F value is the number of degrees of freedom for: the first classification variable (n − 1), the second classification variable (m − 1) or the interaction ((n − 1) × (m − 1)). The second subscript is the number of residual degrees of freedom (Total number of observation − nxm). All results are expressed as mean ± s.e.m.

RESULTS

Characterization of Antisense Targeting Vehicle

Prior to in vivo studies, the asialoglycoprotein-poly(L) lysine: antisense complex and its precursors were characterized by: (1) physicochemical and enzymatic techniques; (2) stability studies of the complexed antisense DNA under a variety of conditions; and (3) in vitro evaluation of hepatocyte uptake and distribution of asialoglycoprotein-poly(L)lysine carrier.

Physicochemical and Enzymatic Analysis of Protein and Antisense Complexes. The characterization of the asialoglycoprotein antisense conjugate began with measurements of the basic physical parameters of the conjugate and its components. The molecular weight of the asialoglycoprotein was measured with a TSK 2000SW column and determined to be about 66,000 daltons. The molar extinction coefficient of the asialo-glycoprotein at 280 nm was $7.2 \times 10^4\ M^{-1}\ cm^{-1}$ (1.09 mg^{-1} ml) as determined using spectrophotometry and the modified Lowry method (*4*). This value did not vary significantly from preparation to preparation over the range of sialic acid cleavage achieved. The molar extinction coefficient of the antisense oligonucleotide at 260 nm was also measured and found to be $1.06 \times 10^6\ M^{-1}\ cm^{-1}$ (1 O.D. unit at 260 nm corresponds to 20 μg/ml).

The asialo-glycoprotein component of the conjugate was generated from human alpha-1 acid glycoprotein by neuraminidase treatment. The number of sialic acid residues released from alpha-1 acid glycoprotein by this treatment was determined in duplicate samples after digestions at 37°C for 1 hr or for an overnight period. Significant differences in the average molar ratios of sialic acid-to-glycoprotein were not detected for the different digestion times: 6.6:1 after 1 hr and 5.3:1 after overnight digestion. These levels of digestion were significantly higher than the value of approximately 1.8:1 reported by Ashwell (*8*) using similar experimental conditions.

The enzymatic and chemical modifications of the glycoprotein were analyzed by HPLC on a TSK 2000SW column. The retention times of the protein standards on this column under the separation conditions used were: IgG: 5.9 min; BSA: 7.2 min; pepsin: 7.7 min; cytochrome C: 8.8 min; and poly(L)lysine: 10.9 min. The calibration curve for this column, (elution time versus log of molecular weight

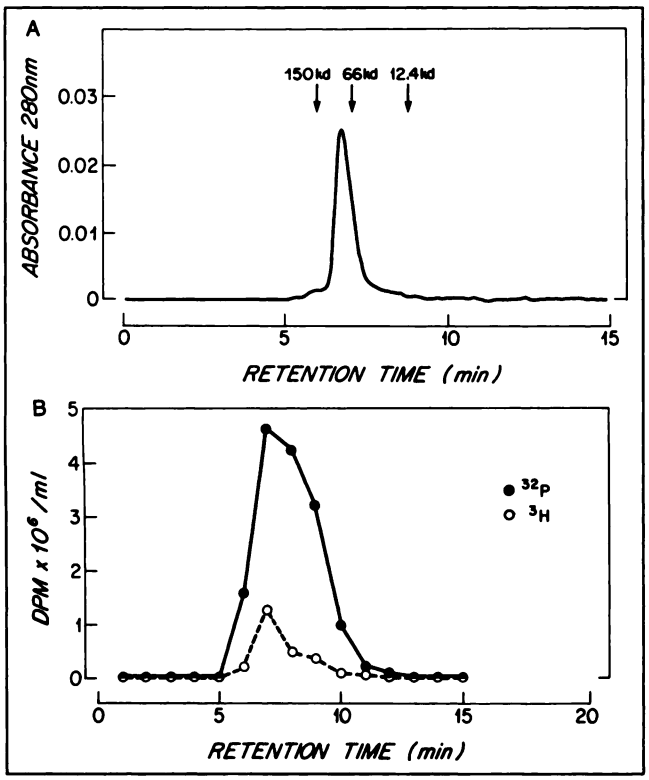

FIGURE 1. Chromatogram of asialoglycoprotein-poly(L)lysine: antisense on a TSK 2000SW column eluted with 0.1 *M* sodium phosphate/0.1 *M* sodium sulfate (pH 6.3) containing 0.02% sodium azide at a flow rate of 1.0 ml/min. (A) Optical density (OD_{280}) tracing; (B) ^{3}H (protein) and ^{32}P (antisense) radioactivity measurements.

standards), was linear and was used to determine the level of poly(L)lysine derivatization of asialoglycoprotein. Under these experimental conditions, the elution times for asialoglycoprotein and asialoglycoprotein-poly(L)lysine were 7.15 and 7.05, yielding by conversion molecular weights of 66kD and 71.4kD, respectively.

Stability of Complexed Antisense DNA. The stability of the asialoglycoprotein antisense complex was monitored by liquid chromatography under various conditions using dual isotope labeling in which the protein was labeled with ^{3}H and the antisense with ^{32}P. An example of a representative chromatogram is shown in Figure 1. As seen in Figures 1A and B, absorption and radioactivity measurements peaked at approximately 7 min after injection which corresponds to the elution time of the intact complex. However, the ratio of $^{32}P/^{3}H$ increased from 4:13 min over the elution time range of 7:9 min, suggesting that at least 30% of the antisense DNA dissociated from the carrier 7 min after injection. When the complex was incubated with PBS, MEM or MEM plus 10% FBS for 1 hr at 37°C at a dilution of 1:100, HPLC analysis demonstrated approximately 85% dissociation. Because of the rapid uptake of the injected conjugate by the liver (greater than 40% of the injected dose is accumulated by the liver within 5 min of injection, see below), this rate of dissociation does not seriously compromise the effectiveness of the conjugate as a targeting vehicle.

The antisense DNA complex was also characterized by agarose gel electrophoresis as described by Wu et al. (*2*). A fixed concentration of ^{32}P-labeled antisense DNA was mixed with varying concentrations of asialoglycoprotein-poly(L)lysine and the molecular species were separated by agarose gel electrophoresis. After autoradiography at −80°C for 1 hr, a molar ratio of 25:1 asialoglycoprotein-poly(L)lysine-to-antisense was found to retard antisense migration on the gel by 95% (Fig. 2).

To visualize the uptake of asialoglycoprotein-poly(L) lysine in rat hepatocytes, poly(L)lysine was labeled with FITC at a molar ratio of 2:1 and covalently linked to asialoglycoprotein using EDAC as described above. The molar ratio of asialoglycoprotein-to-poly(L)lysine-to-FITC in the purified conjugate was determined to be 1:2:2 by chromatography and UV spectroscopy. Figure 3 shows a representative confocal micrograph of rat hepatocytes incubated with asialoglycoprotein-poly(L)lysine-FITC. From this micrograph, it is apparent that the fluorescence intensity is greater in the nuclei than in the cytoplasm.

These analyses indicate that an antisense DNA-carrier protein complex has been prepared and its stability over the time course of hepatic localization has been verified. Also, the complex appears to be internalized by cultured hepatocytes and may localize to the cell nucleus.

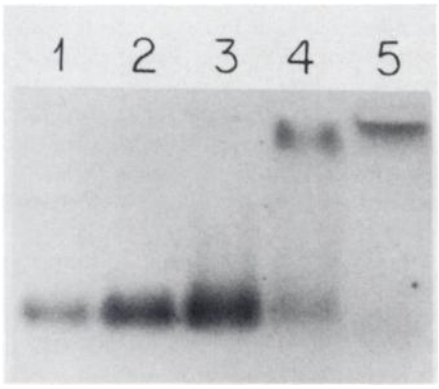

FIGURE 2. Autoradiograph of an agarose gel (10 cm) prepared by mixing a fixed concentration of ^{32}P-labeled antisense DNA with varying concentrations of asialoglycoprotein-poly(L)lysine. The gel was run at 150 volts for 90 min, dried and developed on photographic film at −80°C for 1 hr. The loadings for the lanes are as follows: lane 1: 225 n*M* of antisense alone; 2: 225 n*M* of antisense with 240 n*M* of asialoglycoprotein poly(L)lysine complex (~1:1); 3: 225 n*M* of antisense with 480 n*M* of the protein conjugate (~1:2); 4: 225 n*M* of antisense and 1920 n*M* of protein (~1:8.5); 5: 225 n*M* of antisense with 3850 n*M* of asialoglycoprotein poly(L)lysine (1:17).

Biodistributions of Labeled Conjugate

Results of the biodistribution experiments using ^{32}P-antisense (AS), asialoglycoprotein-poly(L)lysine-to-^{32}P-antisense (ASGP-AS), and ^{3}H sialoglycoprotein-poly(L) lysine-to-^{32}P-antisense (SGP-AS) are shown in Figure 4 (%ID/g tissue). When the data were expressed as %ID/ organ, the pattern and time course of distribution was similar (data not shown). In all experiments, the animals tolerated intravenous administration of the radiolabeled reagents with no apparent toxicity.

As can be seen, the asialoglycoprotein antisense conjugate was rapidly and preferentially taken up by the liver, with a concentration of ~6% ID/g after only 5 min; this concentration did not change significantly at 1 hr. For the sialoglycoprotein conjugate, the corresponding concentrations were 3.5% at 5 min and 2.8% at 1 hr ($p < 0.05$). For antisense alone, the 5-min value was ~1.8% and the 1-hr value was ~2.5% ($p < 0.05$). The spleen and lungs also demonstrated a significant ($p < 0.01$) preferential accumulation of the asialo-glycoprotein conjugate when compared with other tissues.

The biodistributions of ^{3}H and ^{32}P radioactivity in double label experiments were also measured (Fig. 5). Only in the blood, testes and brain was the concentration of labeled protein greater than the concentration of labeled antisense ($p < 0.01$). This indicates that either the effective intracellular half-life of the antisense is greater than that of the glycoprotein for the rest of the organs, or that these organs can accumulate antisense which has dissociated from its carrier molecule while in the circulation.

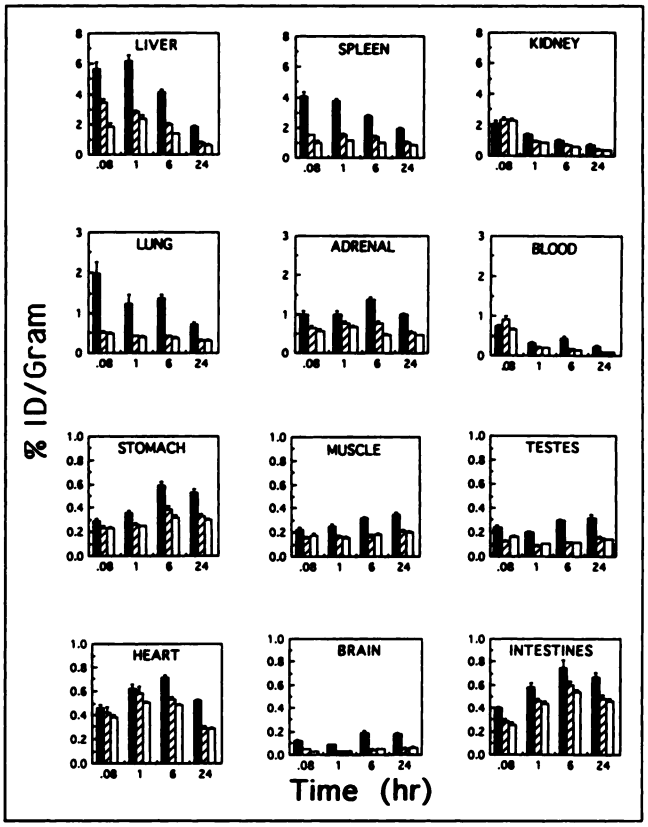

FIGURE 4. Biodistribution of ^{32}P-antisense (AS, open bars), Asialoglycoprotein-poly(L)lysine: ^{32}P-antisense (ASGP-AS, solid bars) and ^{3}H-sialoglycoprotein-poly(L)lysine: ^{32}P-antisense (SGP-AS, hatched bars) in normal rats expressed as percent of injected dose per gram tissue weight. Each point is the mean ± s.e.m. for four to six animals.

DISCUSSION

The use of antisense oligodeoxynucleotides for the regulation of gene expression has become well established in cell culture. However, these systems generally achieve the necessary high intracellular antisense concentrations via micro-injection or via simple addition to cell culture media at near toxic levels. These methods are obviously inappropriate for in vivo applications using antisense molecules, but at present, there are no accepted methods of targeted antisense delivery in vivo. In this paper, we have demonstrated the feasibility of delivering antisense to a specific tissue by coupling the oligonucleotide to a carrier molecule.

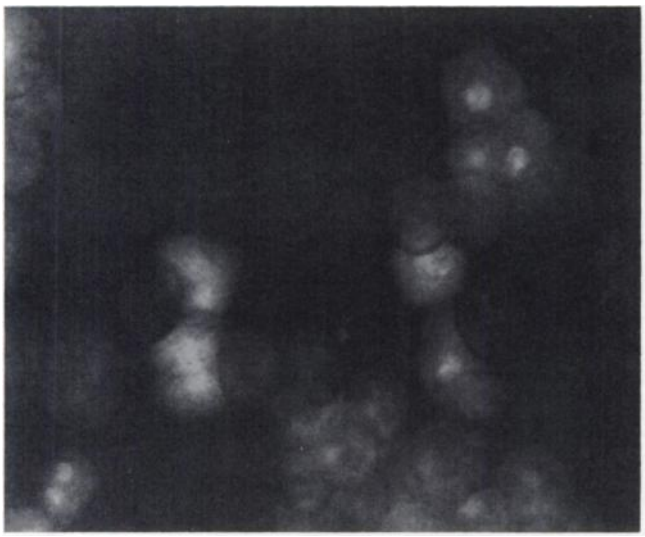

FIGURE 3. Representative confocal micrograph of rat hepatocytes incubated with asialoglycoprotein-poly(L)lysine-FITC, demonstrating increased fluorescence in nuclei compared to the cytoplasm.

The asialoglycoprotein-poly(L)lysine-antisense complex prepared in the current study was chemically similar to the reagent used in previous studies. The use of asialoglycoprotein-poly(L)lysine derivatives with high molecular weight poly(L)lysine polymers (MW: 69,000) and high loading (5:1) for in vitro studies has been reported by Wu et al. (*2*). However, low molecular weight polymers (MW: 3,800) and loadings (2:1) have been more useful for in vivo investigations (*5*).

Characterization of the sialoglycoprotein and asialoglycoprotein reagents established that the number of sialic acid residues cleaved by neuraminidase was about six per protein, and that the stability of the antisense protein conjugate was acceptable given the kinetics of asialoglycoprotein uptake by the liver. Overall, the results of the sialic acid assay and the chromatographic behavior of asialogly-

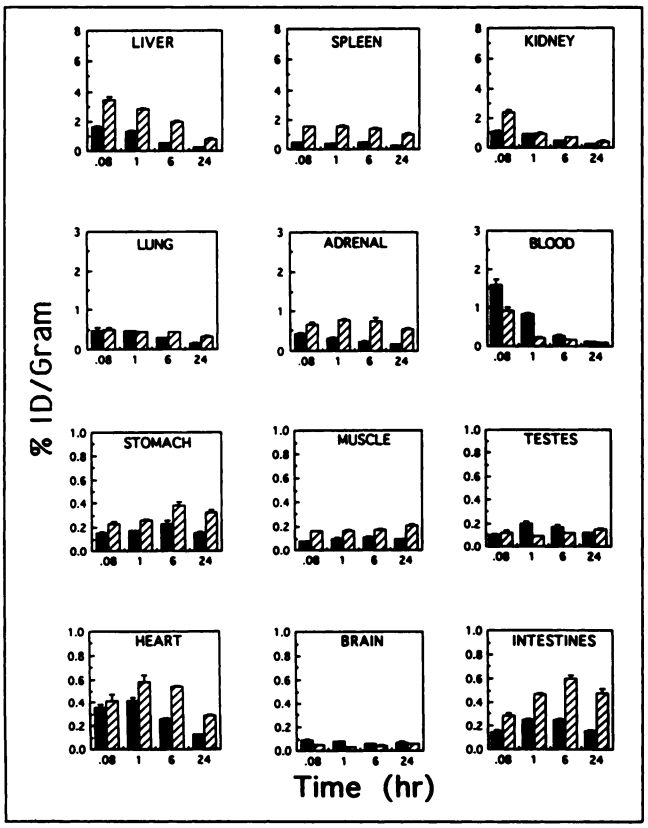

FIGURE 5. Biodistribution of ^{3}H (solid bars) and ^{32}P (hatched bars) radioactivity after injection of ^{3}H-sialoglycoprotein-poly(L) lysine: ^{32}P-antisense in normal rats expressed as percent injected dose per gram tissue weight. Each point is the mean ± s.e.m. for four to six animals.

coprotein-poly(L)lysine indicate that the hepatocyte targeting reagent that we prepared is similar to the compound used by Wu et al. (*8*) for in vivo studies.

One concern about this targeting system is that although the conjugate might be efficiently accumulated by the liver, it could be sorted to the lysosomes and digested after internalization. To investigate this possibility, we used confocal microscopy to follow the distribution of the conjugate after it was internalized by hepatocytes. Rat hepatocytes incubated with asialo-glycoprotein-poly(L)lysine-FITC were studied and the localization of the labeled antisense was determined. It is clear from these experiments that there is higher fluorescent intensity in the nuclei of the cells than in the cytoplasm, suggesting that a significant amount of asialoglycoprotein-poly(L)lysine escapes fusion with lysosomes and rapidly penetrates into the nucleus of hepatocytes. Although this evidence does not establish that the antisense is not degraded, these results indicate that at least some of the complex can escape from lysosomal digestion.

The biodistribution pattern of asialoglycoprotein-poly (L)lysine antisense is consistent with a mechanism of specific uptake of the conjugate by the liver. From as early as 5 min after injection, the level of hepatic accumulation of this protein is approximately twofold greater than the sialoglycoprotein analog. The level of hepatic accumulation of sialoglycoprotein-poly(L)lysine antisense is only slightly greater than that of the unmodified antisense DNA. Although a similar pattern was observed in most of the other tissues that were analyzed, the total level of protein accumulation was significantly lower in nonhepatic tissues. Accumulation in the spleen and in the brain was approximately 50% and less than 0.4% of the hepatic accumulation, respectively. Accumulation in RES organs is unlikely to be related to particle size since the molecular weight of the complex was shown to be ~71 kD. Also, after incubation with MEM or MEM plus FBS, the size of the complex did not increase. The accumulation of sialo- and asialoglycoprotein-poly(L)lysine antisense in the other tissues might be related to the presence of glycoprotein-binding proteins in other tissues. There have been reports of glycoprotein binding proteins in blood (*10–14*), lung (*15–17*), spleen (*18*), testis (*19*), brain (*20–22*) and the gastrointestinal tract (*23*). Unfortunately, data on the specificity of these receptor systems is unknown.

When tissue levels of glycoprotein (^{3}H radioactivity) and antisense DNA (^{32}P radioactivity) were compared, a greater concentration of ^{32}P was detected in all tissues with the exception of blood, testis and brain. There are several possible explanations for this observation, the most likely being: (1) intracellular degradation of the complex followed by rapid penetration of the antisense DNA into the nucleus; or (2) cellular accumulation of unmodified antisense DNA following dissociation in the circulation. The higher concentration of ^{3}H in the blood is consistent with our observation that there is significant dissociation of the antisense from the conjugate. The relative excess of ^{3}H radioactivity in the blood is greater at the early time points after injection than at the 6- and 24-hr readings. This might be due to a process of uptake of antisense DNA by circulating cells. However, the distribution of radioactivity between the plasma and the cellular elements of the blood was not evaluated in this study.

Although a nontrivial amount of the injected antisense DNA conjugate (up to 50% of the dose) distributed to nontarget organs, this should not seriously hinder the efficacy of therapeutic effects. This is due to the fact that therapy with receptor targeted antisense DNA has dual selectivity. Even though the complex can distribute to and possibly be internalized by nontarget organs, biological effects will be elicited only in tissues that express the gene sequence that is complementary to the antisense oligonucleotide. This is in marked contrast to therapy with radiolabeled or toxin conjugated monoclonal antibodies where the therapeutic moiety can have adverse effects on normal tissues.

The results of this study establish that the asialoglycoprotein moiety is an effective vector for targeting antisense oligonucleotides to the liver. This approach may be useful for both diagnostic and therapeutic applications, including the modulation of the expression of hepatic secretory proteins involved in inflammation and the inhibition of the expression of oncogenes in various types of primary liver tumors.

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