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Research Paper

Intravenous siRNA of Brain Cancer with Receptor Targeting and Avidin–Biotin Technology

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Purpose. The effective delivery of short interfering RNA (siRNA) to brain following intravenous administration requires the development of a delivery system for transport of the siRNA across the brain capillary endothelial wall, which forms the blood–brain barrier *in vivo*.
Methods. siRNA was delivered to brain *in vivo* with the combined use of a receptor-specific monoclonal antibody delivery system, and avidin–biotin technology. The siRNA was mono-biotinylated on either terminus of the sense strand, in parallel with the production of a conjugate of the targeting MAb and streptavidin.
Results. Rat glial cells (C6 or RG-2) were permanently transfected with the luciferase gene, and implanted in the brain of adult rats. Following the formation of intra-cranial tumors, the rats were treated with a single intravenous injection of 270 μg/kg of biotinylated siRNA attached to a transferrin receptor antibody via a biotin–streptavidin linker. The intravenous administration of the siRNA caused a 69–81% decrease in luciferase gene expression in the intracranial brain cancer *in vivo*.
Conclusions. Brain delivery of siRNA following intravenous administration is possible with siRNAs that are targeted to brain with the combined use of receptor specific antibody delivery systems and avidin–biotin technology.

KEY WORDS: avidin; biotin; blood–brain barrier; monoclonal antibody; RNA interference.

INTRODUCTION

RNA interference (RNAi) enables the knockdown of target gene expression with either short interfering RNA (siRNA) or short hairpin RNA (shRNA) (1). The latter are generally expressed within the target cell following transfection with plasmid DNA encoding the shRNA (2). RNAi in target cells in culture is routine with the use of cationic polymers as the delivery agent. The complex of the anionic siRNA and the cationic polymer leads to aggregation in saline (3), and the aggregation triggers phagocytosis of the nucleic acid complex in cultured cells (4). While aggregation is acceptable in cell culture, aggregates may embolize in the pulmonary microcirculation *in vivo*. Therefore, it is important to develop *in vivo* siRNA delivery systems, which avoid the requirement for electrostatic complexes between anionic DNA and a cationic polymer.

One approach to the *in vivo* delivery of RNAi drugs is the use of molecular Trojan horses, which are receptor-specific monoclonal antibodies (MAb) (5). An endocytosing MAb to the transferrin receptor (TfR), or the insulin receptor, can deliver the therapeutic to the intracellular space of the target cell. The problem is how to formulate the complex of the targeting MAb and the siRNA drug in such a way that the complex is stable *in vivo* in the circulation. In prior work, a plasmid DNA encoding a shRNA against the human epidermal growth factor receptor (EGFR) was combined with MAbs to the mouse transferrin receptor (TfR) and the human insulin receptor (HIR) using pegylated immunoliposomes (PILs), also called Trojan horse liposomes (6). The plasmid DNA was encapsulated in the interior of the PIL, and the tips of the polyethyleneglycol polymers on the surface of the PIL were tethered with the receptor-specific MAb (6). The weekly administration of PILs to mice with intra-cranial human brain cancer led to a 90% increase in survival time, which was mediated via shRNA knockdown of the EGFR (6).

The purpose of the present investigation is to extend the molecular Trojan horse delivery technology to siRNA drugs. In this case, liposome technology is replaced with avidin–biotin technology. The siRNA is mono-biotinylated in parallel with the production of a 1:1 conjugate of streptavidin (SA) and the targeting MAb. Owing to the very high affinity of SA binding of biotin, which has a $K_D=10^{-15}$ M and a dissociation $t_{1/2}$=89 days (7), there is immediate capture of the biotinylated siRNA upon mixing with the MAb/SA conjugate. Among the initial problems to be addressed in the present investigation was whether a siRNA could be mono-biotinylated and conjugated to the 50,000 Da streptavidin, and still retain RNAi activity. The present

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study follows on prior work showing that a peptide nucleic acid (PNA) antisense agent could be biotinylated and bound to the MAb/SA conjugate, and still hybridize to the target mRNA (8).

MATERIALS AND METHODS

Materials

Male Fischer CD344 rats were purchased from Harlan Breeders (Indianapolis, IN). The U87 human glioblastoma and RG2 rat malignant glioma cell lines were purchased from the American Type Tissue Collection (ATCC, Manassas, VA). MEM, DMEM, fetal bovine serum (FBS), Lipofectamine 2000, and Lipofectamine were purchased from Invitrogen (Carlsbad, CA). Recombinant streptavidin (SA) and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The low molecular weight DNA duplex ladder was from New England Biolabs (Ipswich, MA). The 8314 murine monoclonal antibody (MAb) to the human insulin receptor, and the OX26 murine MAb to the rat transferrin receptor (TfR) were purified from ascites by protein G affinity chromatography. The HIRMAb is active only in humans (9), and the TfRMAb is active only in rats (10,11). The 1:1 conjugate of SA and the HIRMAb, designated HIRMAb/SA, and the 1:1 conjugate of SA and the TfRMAb, designated TfRMAb/SA, were produced with a thiol–ether linkage as described previously (12,13). The luciferase expression plasmid DNA is clone 790 described previously (14). Rat C6 glial cells permanently transfected with clone 790 were described previously (14). For this study, rat RG2 glial cells were permanently transfected with clone 790 plasmid DNA with Lipofectamine 2000 and selected with 300 μg/mL of hygromycin B, as described previously (14). GTP enzyme activity was measured with a spectrophotometric enzymatic assay using a kit from Diagnostic Chemicals LTD (Charlottetown, Prince Edward Island, Canada).

The 3′-biotin siRNA, the 5′-biotin siRNA and the non-biotinylated-siRNA were directed at the same sequence of the luciferase mRNA (Fig. 1), and were custom synthesized by Dharmacon (Lafayette, CO); the biotinylated siRNAs contain a 15-atom tetra-ethyleneglycol spacer between the biotin carboxyl group and the 3′- or 5′-hydroxyl group on the siRNA. Single stranded RNA or siRNA duplexes were obtained 2′-deprotected from the vendor. The non-biotinylated siRNA and the 5′-biotinylated siRNA were received from the vendor pre-annealed, and these were dissolved to a final concentration of 70 μM (~1 mg/ml) in annealing buffer (0.1 M potassium acetate, 30 mM HEPES pH=7.2, 2 mM Mg acetate). Following incubation on ice for 15 min, the concentration of the siRNA solution was confirmed by OD260 reading. Because Dharmacon does not provide annealed siRNA with the 3′-biotin modification, the single stranded luciferase antisense and 3′-biotinylated sense RNAs were individually synthesized. Both sense and antisense RNA were dissolved in annealing buffer at a final concentration of 140 μM. The single stranded RNAs were pre-treated by incubation for 15 min on ice followed by 10 min at 60 °C and by cooling on ice for 5 min. Equimolar concentrations of sense and antisense RNAs were mixed and annealed for 45 min at 60 °C and 30 min at room temperature to form a 70 μM solution of the 3′-biotin siRNA.

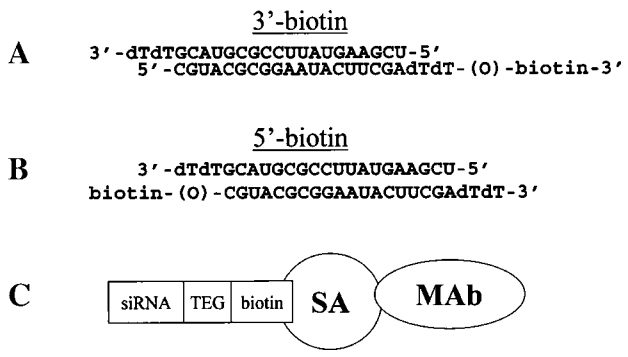

Fig. 1. (**A**) Sequence of anti-luciferase siRNA with a biotin group conjugated to the 3′-terminus of the sense strand. The sequence targeting the luciferase mRNA derived from the pGL2 plasmid is from Elabashir *et al.* (27). (**B**) Sequence of anti-luciferase siRNA with a biotin group conjugated to the 5′-terminus of the sense strand. (**C**) Structure of biotinylated siRNA conjugated to targeting MAb via the biotin streptavidin (SA) linker. TEG: 15-atom tetra-ethyleneglycol spacer between the biotin and the siRNA

Cell Culture and *In Vitro* RNAi

U87 human glioma cells were grown in 6-well cluster dishes with MEM medium containing 10% fetal bovine serum (FBS), and were transfected with clone 790 plasmid DNA. Clone 790 is a pCEP4 expression plasmid encoding the luciferase open reading frame, which was derived from the pGL2 plasmid (14). The sequence and structures of the siRNAs targeting the luciferase mRNA are given in Fig. 1. Stock solutions of the siRNA were prepared in RNAse-free 0.1 M potassium acetate/30 mM Hepes/pH=7.2/2 mM magnesium acetate, and were quantitated with measurements of OD260. One of two experimental designs was used depending on whether the siRNA was delivered to the cell with Lipofectamine or the HIRMAb/SA delivery system. In the first experimental design, the growth medium was removed and replaced by 0.7 mL/well of serum free MEM containing 1 μg of clone 790 plasmid DNA, 2.5 μL Lipofectamine, and 50 pmol of 3′-biotin siRNA, 5′-biotin siRNA, or non-biotinylated siRNA. The dishes were incubated for 4 h at 37 °C. The medium was aspirated and replaced with 1 mL/well of MEM with 10% FBS. After 48 h of incubation, the medium was aspirated, the monolayer was washed with PBS, and the cell lysate was prepared in luciferase lysis buffer (Promega, Madison, WI), where PBS = 0.01 M Na_2HPO_4/0.15 M NaCl/pH=7.4. Luciferase activity was measured with a luminometer, cell protein was measured with the bicinchoninic acid (BCA) assay (Pierce Chemical Co, Rockford, IL), and the luciferase activity was reported as pg luciferase per mg protein, as described previously (15). In the second experimental design, the growth medium was removed and replaced by 0.7 mL/well of serum free MEM containing 1 μg of clone 790 plasmid DNA and 2.5 μL Lipofectamine without siRNA. After a 4 h incubation at 37 °C, the medium was aspirated and 0.7 mL/well of MEM containing the siRNA (50 pmol/dish) with 0 or 100 pmol/dish of HIRMAb/SA was added. After 48 h, the medium was discarded, and the cellular luciferase enzyme activity was measured.

siRNA Agarose Gel Electrophoresis and Size Exclusion HPLC

The attachment of the biotinylated siRNA to the MAb/SA conjugate was verified with an agarose gel shift technique. The 5′-biotin siRNA, the 3′-biotin siRNA, and the non-biotinylated siRNA (20 pmol) were loaded in 1 μL volumes onto 4% NuSieve GTG agarose gels (FMC, Rockland, ME) with the ethidium bromide (16). The samples were diluted with 0.5× TBE buffer (Sigma) and 2 mg/ml Orange G (Sigma). Electrophoresis was performed in 0.5× TBE buffer at 80 V for 1.5 h. The RNA bands were visualized under ultraviolet light and the gel was photographed. The molecular size of the HIRMAb/SA conjugate and the complex of the HIRMAb/SA conjugate and the biotinylated siRNA was measured with a PSS-Nicomp Particle Sizing Systems Model 380 Submicron Particle Analyzer (Santa Barbara, CA).

The conjugation of the biotinylated siRNA to the TfRMAb/SA conjugate was confirmed by size exclusion chromatography (SEC) using a 7.8 mm×30 cm (5 μm) G2000SW high pressure liquid chromatography (HPLC) column (Tosoh Bioscience LLC, Montgomeryville, PA), and a Perkin-Elmer Series 200 pump and UV/VIS detector. The 5′-biotin siRNA either alone, or conjugated to TfRMAb/SA, or TfRMAb/SA without attached siRNA, was injected on to the column in a 200 μL volume of PBS, followed by elution in PBS at a flow rate of 0.5 mL/min with detection at 260 nm. The elution profile was recorded with a Servocorder model SR6253 chart recorder (Datamark Corp., Costa Mesa, CA), at 0.5 cm/min, and the chart paper was scanned with a UMAX PowerLook III scanner (Techville, Inc., Dallas, TX) into Adobe Photoshop.

In Vivo Brain Cancer Model

Male Fischer CD344 rats weighing 180–200 g were anesthetized with ketamine/xylazine, and a burr hole was drilled 3 mm to the right of midline and 1 mm posterior to bregma. Either C6 cells permanently transfected with clone 790, and designated C6-790 cells, or RG-2 cells permanently transfected with clone 790, and designated RG2-790 cells, were suspended in serum-free MEM containing 1.2%methyl cellulose. Cell suspensions (5 μL; 500,000 cells) were injected into the right caudate-putamen nucleus at a depth of 4 mm over a 2 min period, using a 10-μL Hamilton syringe with fixed needle, as described previously (15), with four tumor bearing rats per treatment group. All animal procedures were approved by the UCLA Animal Research Committee. At 4 days after the implantation, the C6 tumor-bearing rats were given a single intravenous (IV) injection of either saline, or 5′-biotinylated-siRNA alone (80 μg/rat), or 5′-biotinylated siRNA (80 μg/rat) conjugated to the TfRMAb/SA (736 μg/rat), and the animals were sacrificed at 6, 24 or 48 h after the IV injection. The luciferase and γ-glutamyl transpeptidase (GTP) enzyme activity in the tumor and contralateral brain extracts were measured with a luminometer, and with an enzymatic spectrophotometric assay, respectively, as described previously (15). In a second study, rats were injected with the RG2-790 cells at day zero in the same region of the caudate-putamen nucleus. At day 4 after implantation, all rats were given a single IV injection of 5′-biotin- siRNA, without or with attachment to the TfRMAb/SA delivery vector. The doses of siRNA and TfRMAb were 68 μg/rat and 810 μg/rat, respectively. The animals were then sacrificed at either 24 or 48 h after the injection, which are 5 or 6 days after the tumor cell implantation, followed by measurement of luciferase and GTP enzyme activity in the tumor and contralateral brain.

Cytokine ELISA

The cytokine response in human U87 glial cells in culture was evaluated with an ELISA specific for human interferon (INF)-α from PBL Biomedical Labs (Piscataway, NJ). The cytokine response in rats was evaluated with an ELISA specific for rat interleukin (IL)-6 from R&D System Inc (Minneapolis, MN). A typical IL-6 ELISA standard curve produced absorbance at 450 nm (A450) of 2.58, 1.23, 0.58, 0.26, 0.13, 0.078, 0.033, and 0, at 4,000, 2,000, 1,000, 500, 250, 125, 65, and 0 pg/mL. All undiluted rat serum samples (50 μL) produced an A450 reading of <0.02.

Statistics

Statistical differences were assessed with Student's *t*-test, or by analysis of variance (ANOVA) with Bonferroni correction.

RESULTS

The structures of the 3′-biotin siRNA and 5′-biotin siRNA are shown in Fig. 1A and B, respectively. The attachment of the siRNA to the MAb delivery system via the biotin-SA bridge is shown in Fig. 1C. The complete conjugation of the biotinylated siRNA to the MAb was verified with an agarose gel shift technique (Fig. 2). Figure 2A shows the following samples: lane 1 is a sizing ladder of DNA duplexes; lane 2 is the non-biotinylated anti-luciferase siRNA duplex; lane 3 is the biotinylated siRNA duplex; lane 4 is the single-stranded anti-luciferase siRNA. The agarose gel in Fig. 2A demonstrates effective formation of the RNA duplex and the slightly larger molecular size of the mono-biotinylated form of the siRNA duplex. The samples in Fig. 2B include the following: lane 2, a conjugate of the HIRMAb/SA and the 3′-biotin siRNA; lane 3, the 3′-biotin-siRNA without conjugation to the MAb; lane 4, conjugate of the HIRMAb/SA and the 5′-biotin siRNA; lane 5, the 5′-biotin siRNA without attachment to the HIRMAb; lane 6, a mixture of the non-biotinylated siRNA and the HIRMAb/SA conjugate which shows no formation of a conjugate owing to absence of the biotin moiety incorporated in the siRNA; lane 7, non-biotinylated siRNA alone. The data in Fig. 2B show that there is 100% incorporation of the biotin moiety into the siRNA with the appropriate shift in size following attachment to the HIRMAb via a SA-biotin linker. Particle analyzer measurements showed the mean diameter of the HIRMAb/SA, without siRNA, was 29.6±1.9 nm, and was 40.6±2.1 nm, with the biotinylated siRNA attached (mean±SE, n=3). On SEC HPLC, the biotinylated siRNA eluted at a volume of 5.7 mL (Fig. 3A) and 7.9 mL (Fig. 3B), following injection of the siRNA either conjugated to TfRMAb/SA or alone, respectively. The TfRMAb/SA, without siRNA, eluted at 5.7 mL (Fig. 3C).

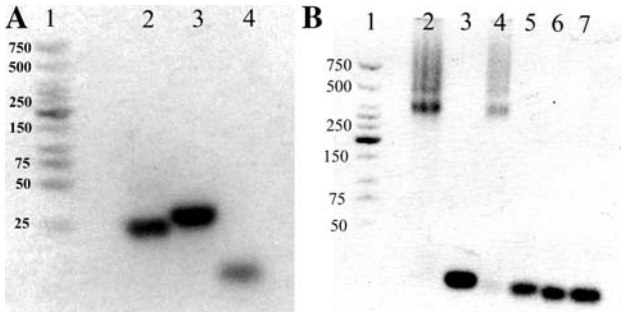

Fig. 2. Ethidium bromide stained agarose gel electrophoresis. (**A**) Lane 1: DNA duplex ladder; the number of base pairs in the standard DNA duplex is shown; Lane 2: non-biotinylated anti-luciferase siRNA duplex; Lane 3: biotinylated anti-luciferase siRNA duplex; Lane 4: non-biotinylated anti-luciferase siRNA sense strand. (**B**) Lane 1: DNA duplex ladder; Lane 2: conjugate of HIRMAb/SA and 3′-biotin siRNA duplex; Lane 3: the 3′-biotin siRNA duplex without the HIRMAb/SA; Lane 4: conjugate of HIRMAb/SA and 5′-biotin siRNA duplex; Lane 5: the 5′-biotin siRNA duplex without the HIRMAb/SA; Lane 6; the non-biotinylated siRNA mixed with the HIRMAb/SA; Lane 7: the non-biotinylated siRNA duplex without the HIRMAb/SA

The delivery of mono-biotinylated siRNA to target cells with a molecular Trojan horse and avidin-biotin technology was initially evaluated in tissue culture with human glial cells. Since it was not known whether biotinylation of the 3′ or 5′ terminus of the sense strand would interfere with siRNA targeting of luciferase RNA, a series of experiments was performed involving co-transfection of human U87 glial cells with both a luciferase expression plasmid, designated clone 790, and the anti-luciferase siRNAs shown in Fig. 1A and B. In parallel, an anti-luciferase siRNA with identical sequence was produced that contained no biotin moiety. As shown in Table I, the co-lipofection of U87 cells with the clone 790 plasmid DNA and the non-biotinylated siRNA resulted in an 80% inhibition of luciferase gene expression in cell culture (Experiment I, Table I). An 87–90% inhibition of luciferase gene expression was observed with the co-lipofection of the luciferase plasmid and the 5′-biotinylated siRNA or the 3′-biotinylated siRNA (Experiment I, Table I). These results demonstrated that biotinylation of either the 5′ or 3′ terminus of the sense strand did not interfere with siRNA activity. The effect of conjugation of the biotinylated siRNA to a large molecule, such as recombinant streptavidin, 50,000 Da molecular weight, on siRNA function was examined next. In these experiments, a complex of the recombinant streptavidin and the 5′- or 3′-biotinylated siRNA was produced prior to mixture with the DNA and lipofectamine for co-lipofection of human U87 glial cells with the luciferase plasmid and the siRNA. As demonstrated in Table I (Experiment II), conjugation of the 5′-biotin siRNA or the 3′-biotin siRNA to recombinant streptavidin did not impair siRNA activity in cell culture.

U87 human glial cells were transfected with clone 790 plasmid DNA using lipofectamine for 4 h without exposure to the siRNA. The cells were then washed and lipofectamine-free 5′-biotin siRNA or 3′-biotin siRNA was added to the medium for a 48 h period either with or without conjugation to the 83-14 HIRMAb via an SA-biotin linker. As shown in Fig. 4, the luciferase expression levels were high in the transfected cells, approximating 200 pg/mg protein. There is no inhibition of luciferase expression following application of the HIRMAb/SA conjugate alone (i.e. without siRNA attached) and there was either minimal or no inhibition of luciferase gene expression following addition of either 5′-biotin siRNA or 3′-biotin siRNA without addition of the HIRMAb delivery system. However, there was a 79–86% inhibition of luciferase gene expression when the 5′-biotin siRNA or the 3′-biotin siRNA was conjugated to the HIRMAb delivery system (Fig. 4). The delivery of the siRNA to the human U87 cells with the HIRMAb did not

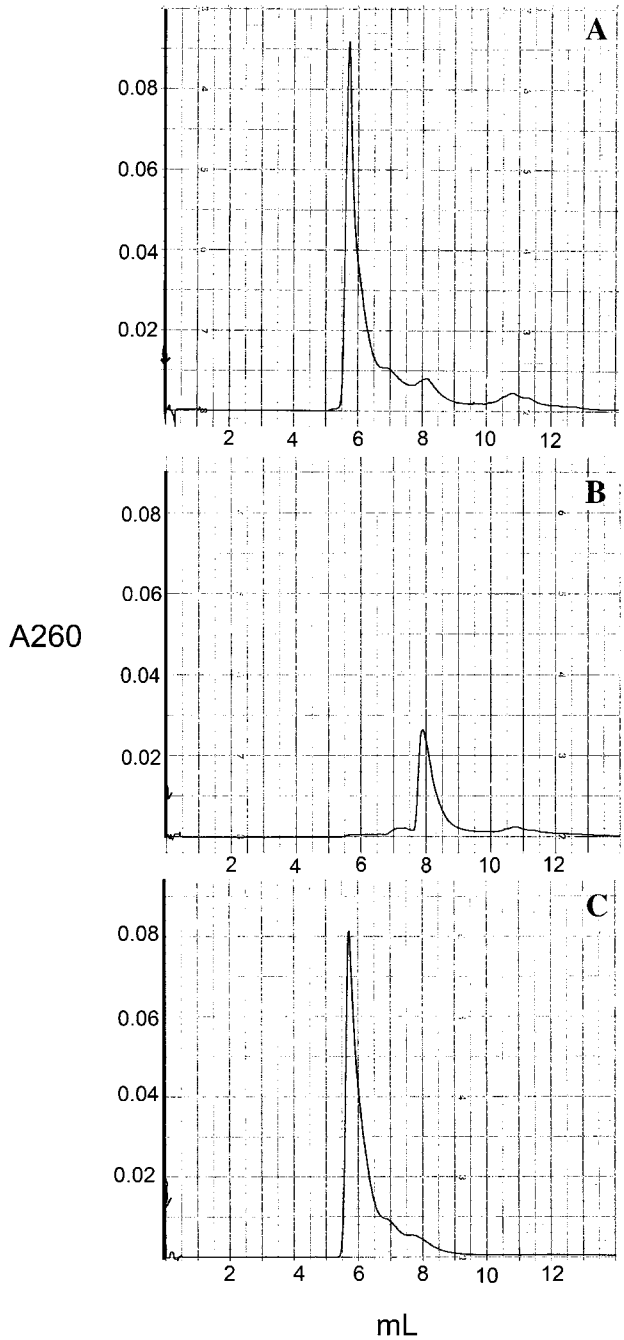

Fig. 3. SEC HPLC of the 5′-biotin siRNA conjugated to OX26/SA (**A**), the unconjugated 5′-biotin siRNA (**B**), and the OX26/SA without attached siRNA (**C**)

Table I. Lipofectamine Co-Transfection of Human U87 Glial Cells with Anti-Luciferase siRNA and Clone 790 Luciferase Expression Plasmid

Experiment	Treatment	Luciferase (pg/mgp)
I	Control	33.6±3.2
	Non-biotin siRNA	6.7±1.0*
	5′-biotin siRNA	4.3±1.6*
	3′-biotin siRNA	3.4±0.4*
II	Control	29.5±8.9
	5′-biotin siRNA	1.4±0.1*
	5′-biotin siRNA + SA	1.8±0.8*
	3′-biotin siRNA	0.65±0.15*
	3′-biotin siRNA + SA	0.97±0.26*

Data are mean±SE (n=4). The following was added to each 35 mm dish (9.5 cm^2): 0.7 mL medium containing 1 μg plasmid DNA, 2.5 μL Lipofectamine, 50 pmol of siRNA (70 nM), and 100 pmol of SA (140 nM). The control solution is the siRNA buffer, 0.1 M potassium acetate/2 mM magnesium acetate/30 mM HEPES/pH=7.4 diluted 25-fold in the final assay medium.
*p<0.01 by ANOVA difference between control. The differences between the non-biotin siRNA and the biotin siRNA in Experiment I are not significantly different.

induce a cytokine reaction, based on measurements of human interferon (INF)-α with a specific ELISA. The assay was sensitive to 100 pg/mL (Methods), and no measurable INF-α was detected in the medium at 6, 24, or 48 h of incubation. Inhibition of luciferase expression with siRNA treatment caused no decrease in cellular GTP enzyme activity in cultured U87 cells.

The *in vivo* delivery of an anti-luciferase siRNA to intracranial brain cancers in adult rats was examined initially with brain tumors formed by C6 rat glioma cells permanently transfected with the clone 790 luciferase expression plasmid. These cells were implanted in the caudate-putamen nucleus of adult Fisher CD344 rats, which led to the formation of intracranial gliomas, as demonstrated previously (15). At 4 days after implantation of 500,000 glial tumor cells, the rats were administered either saline or the 5′-biotin siRNA conjugated to the OX26 TfRMAb via the SA-biotin linker. A 69% (p<0.05) inhibition in tumor luciferase expression was observed at 48 h following a single intravenous administration of the 3′-biotin anti-luciferase siRNA conjugated to the TfRMAb (Fig. 5A). The decrease in luciferase gene expression in the C6 tumor model was not a non-specific effect, as there was no inhibition of γ-glutamyl transpeptidase (GTP) expression in either the C6 tumor or the contra-lateral brain in the siRNA treated animals (Table II).

The C6 tumor may cause an immune reaction in rat brain (17). However, there is no immune reaction following implantation of the syngeneic RG-2 tumor cells in the rat (17). Therefore, rat glial RG-2 cells were permanently transfected with the clone 790 luciferase expression plasmid and transfectants were selected with hygromycin B (Methods). The RG-2 cells were then implanted in the caudate-putamen nucleus of the brain of adult Fisher CD344 rats. At 5 days after implantation of 500,000 tumor cells, the rats were injected intravenously with either 5′-biotin siRNA without conjugation to the TfRMAb, or 5′-biotin siRNA conjugated to the TfRMAb through an SA-biotin linker. An 81% (p<0.05) inhibition of luciferase expression was observed in the RG-2 intracranial brain tumor at 48 h following a single intravenous administration of the biotin-siRNA, providing the RNAi therapeutic was conjugated to the TfRMAb (Fig. 5B). GTP enzyme activity was measured in both tumor and contralateral brain in both treatment groups shown in Fig. 5B, and there were no significant differences in GTP enzyme activity. No cytokine response was detected *in vivo* as the serum interleukin (IL)-6 level was <65 pg/mL at 6 and 24 h after siRNA injection using an ELISA specific for rat IL-6 (Methods).

DISCUSSION

The results of these studies are consistent with the following conclusions. First, siRNAs can be mono-biotinylated on either the 5′- or 3′-terminus of the sense strand of the siRNA, bound to SA, and still retain RNAi activity (Table I). Second, cell culture siRNA delivery agents such as lipofectamine can be replaced with an antibody based delivery system, wherein the siRNA and MAb are connected via a biotin-SA linker (Fig. 1C), and the RNAi effects are retained (Fig. 4). Third, the combined use of biotinylated siRNAs, receptor-specific molecular Trojan horses, and avidin-biotin technology enables intravenous RNAi *in vivo* in an experimental intra-cranial brain cancer model (Fig. 5). Fourth, antibody-mediated delivery of siRNA has no effects on cytokine response (Results) or on non-target genes (Table II).

Ligands, such as biotin, could be placed on either the 5′- or 3′-terminus of either the sense or antisense strand of the siRNA duplex. However, prior work has shown that RNAi effects are lost when the 5′-terminus of the antisense strand is altered (18,19). Therefore, the present work evaluated placement of the biotin/SA linkage at the 3′- or 5′-terminus of the sense strand (Fig. 1). The results show that neither the 3′- nor 5′-biotin, with or without the attached SA, has any effect on RNAi activity (Table I). This finding parallels prior work showing that a PNA antisense agent still hybridizes to the target mRNA following biotinylation of the PNA, and attachment to the MAb/SA delivery system (8). The retention of biological activity of the siRNA following biotinylation, and attachment to SA, allows for high affinity attachment of the

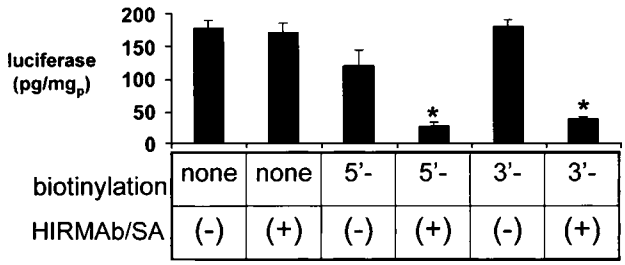

Fig. 4. Luciferase gene expression in human U87 glial cells in culture. The cells were grown in 35 mm dishes, and were transfected with 1 μg/dish of luciferase expression plasmid (clone 790) and 2.5 μL/dish of Lipofectamine for 4 h. The medium was replaced with 0.7 mL/dish of serum free MEM containing 50 pmol/dish of the anti-luciferase siRNAs (70 nM), which were pre-mixed either without (−) or with (+) conjugation to 100 pmol/dish of 83-14 HIRMAb/streptavidin (SA) (140 nM). The anti-luciferase siRNA was produced in three formulations: (1) no biotin, (2) 5′-biotin (Fig. 1B), or (3) 3′-biotin (Fig. 1A). The conjugation of the 5′-biotin or the 3′-biotin-siRNA to the HIRMAb/SA resulted in an 79–86% knockdown in luciferase gene expression. *p<0.01 difference from control (no biotinylation; no HIRMAb/SA) by ANOVA

mono-biotinylated siRNA to a conjugate of SA and a receptor-specific targeting ligand, such as the HIRMAb or the TfRMAb. The mean diameters of the HIRMAb/SA conjugate, without attached siRNA, and the complex of the siRNA and the HIRMAb/SA conjugate, depicted in Fig. 1C, are 29.6±1.9 nm and 40.6±2.1 nm, respectively (Results). This sizing correlates with the gyration diameter of an IgG molecule, which is 18 nm (20), which represents the size of the IgG without any attached SA or biotinylated siRNA duplex.

In the absence of lipofectamine, the addition of the siRNA to tissue culture medium has minimal RNAi effect (Fig. 4). The addition of the cationic lipid is essential to RNAi activity in cell culture, because the formation of the complex between the anionic siRNA and the cationic lipid causes the formation of aggregates, which triggers uptake by the cell. The aggregation of siRNA and cationic lipid is not observed in low salt solution, but is induced in physiological saline (3). The aggregation of siRNA and cationic polymer, such as polyethyleneimine, may not be observed *in vitro* in physiological saline, but is observed *in vivo* (21). Such aggregation may cause embolization in the pulmonary circulation following intravenous administration, which has been shown previously for anionic plasmid DNA and cationic polyplexes (22). While such aggregation may facilitate siRNA uptake into the lung endothelium *in vivo* (23), or in flank tumors (21), this property makes it difficult to deliver siRNA to the brain following intravenous administration. It is not possible to achieve RNAi *in vivo* in distant organs such as the brain with cationic polymers alone (21). An alternative approach is to link the siRNA to a BBB delivery system. A siRNA was complexed to poly-arginine, which was fused to a 29-amino acid peptide derived from the rabies virus glycoprotein (RVG) (24). The RVG peptide is hypothesized to cross the BBB via receptor-mediated transport on the brain capillary endothelial nicotinic acetylcholine receptor (AChR) (24). However, activation of brain microvascular AChR causes BBB disruption (25).

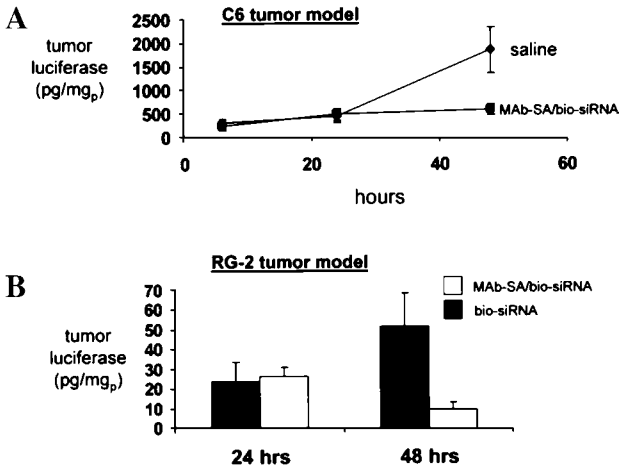

Fig. 5. Luciferase gene expression in intra-cranial C6 rat gliomas (**A**) or RG2 rat gliomas (**B**) is knocked down by intravenous siRNA, providing the RNAi is conjugated to the TfRMAb via a biotin–SA linker. In this model, cultured C6 rat glioma cells, or RG-2 rat glioma cells were permanently transfected with a luciferase expression plasmid. Following selection, the tumor cells were implanted in the brain of Fischer CD344 rats; 5 days later, the animals were injected with either saline, 5′-biotinylated anti-luciferase siRNA alone (bio-siRNA) or 5′-biotinylated anti-luciferase siRNA conjugated to the TfRMAb via the biotin–SA linker (MAb-SA/bio-siRNA). Intravenous RNAi-mediated knockdown of brain tumor luciferase activity is observed only when the siRNA is attached to the TfRMAb. The dose of siRNA in this study is 68 μg/rat or 270 μg/kg as a single intravenous injection at zero time. The inhibition at 48 h in luciferase expression is 69% in the C6 tumor model ($p<0.05$), and is 81% in the RG-2 tumor model ($p<0.05$)

Table II. γ-Glutamyl Transpeptidase (GTP) Enzyme Activity in C6-790 Brain Tumor *In Vivo*

Time (h)	Treatment	Tumor GTP	Contralateral brain GTP
6	Saline	7.2±0.4	1.7±0.2
	siRNA-MAb	6.5±0.9	1.8±0.3
24	Saline	3.7±0.6	1.4±0.3
	siRNA-MAb	3.9±1.0	1.7±0.3
48	Saline	2.6±0.5	1.6±0.3
	siRNA-MAb	3.1±0.4	2.1±0.6

Data are mean±SE (n=3 rats per group). There are no significant differences between the saline and siRNA treated groups. The siRNA-MAb conjugate is the same as the bio-siRNA/SA-MAb in Fig. 5A.

The present study investigates whether siRNA can be delivered through the BBB via the brain endothelial TfR. Prior work has shown that antisense agents, such as PNAs, can be delivered to brain *in vivo* via the BBB TfR, following attachment of the biotinylated PNA to a conjugate of SA and an MAb to the rat TfR (8,12). This TfRMAb is not active in human cells, so the delivery of the siRNA to cultured human U87 cells was investigated in the present study with the HIRMAb. As shown in Fig. 4, there is a 79–86% knockdown of luciferase gene expression in these cells following delivery of the 3′- or 5′-biotin siRNA with the HIRMAb delivery system. The agarose gel shift assay (Fig. 2), and the SEC HPLC (Fig. 3), show attachment of >90% of the biotinylated siRNA to the HIRMAb/SA (Fig. 2), and TfRMAb/SA (Fig. 3) delivery system, respectively. The HIRMAb is not active against the rodent insulin receptor (9), so the *in vivo* RNAi studies in the rat brain tumor model were performed with the conjugate of SA and the OX26 MAb against the rat TfR.

The rat TfR is expressed on both the tumor vascular endothelial cell, which forms the BBB *in vivo*, and on the tumor cell membrane in the rat brain tumor model (6,15). Therefore, *in vivo* RNAi in the intra-cranial brain tumor model requires that the siRNA traverse 2 membranes in series, the BBB, which is comprised of the lumen and ablumenal endothelia membranes, and the tumor cell plasma membrane. A 69–81% knockdown of target luciferase gene expression was observed at 48 h after the single intravenous administration of the siRNA, providing the siRNA was complexed to the TfRMAb delivery system (Fig. 5). No suppression of luciferase gene expression was observed at 24 h after administration (Fig. 5). This result parallels the findings of another RNAi *in vivo* study, where a siRNA against caveolin-1 was delivered to lung capillary endothelium with cationic lipid, dimethyldioctadecylammonium bromide (DDAB). Caveolin-1 gene knockdown *in vivo* was not observed until 48 h after intravenous administration administration of 1,300 μg/kg of the siRNA/DDAB complex (23).

The intravenous dose of siRNA that produced the suppression of tumor luciferase expression in the present study was 68 μg/rat or 270 μg/kg, which was administered 4 days after implantation of 500,000 cells in rat brain. By this time, the tumor is large and occupies nearly the entire volume of the striatum in the rodent brain, as demonstrated by a prior study from this laboratory (15). The tumor formation in the present study is verified by the high brain luciferase enzyme activity in the untreated animals (Fig. 5). In the animals treated with siRNA conjugated to the TfRMAb, there was a 69–81% inhibition in tumor luciferase enzyme activity (Fig. 5). Therefore, intravenous RNAi with targeted siRNA using avidin-biotin technology is effective with delayed administration at low systemic doses of the siRNA. The SA-biotin linkage is stable *in vivo* as demonstrated previously by gel filtration chromatography of serum removed 60 min following administration in rats or mice (8,26). The stability of the linkage between the siRNA and the targeting MAb is derived from the very high affinity of biotin binding to SA, which is characterized by a K_D of 10^{-15} M and a dissociation $t_{1/2}$ of 89 days (7). Given these kinetics, the SA-biotin bond is highly suited for pharmaceutical delivery *in vivo*. However, apart from the stability of the SA-biotin bond, the other source of instability of the complex *in vivo* is degradation of the siRNA duplex, which is susceptible to both 3′-exonuclease and endonuclease. The siRNAs used in the present study contain 2-nucleotide 2′-deoxythymidine (dT, Fig. 1) overhangs to suppress exonuclease activity (27). However, the siRNA is still subject to endonuclease activity, and future formulations may include 2′-*O* nucleic acid substitutions, which promote nuclease resistance (28). The attachment of the nucleic acid to the MAb/SA complex does not impair interaction with the BBB TfR receptor, as the BBB permeability coefficient of the TfRMAb, a complex of neutral avidin and the TfRMAb, and a complex of a PNA, SA, and the TfRMAb, are all approximately the same (12,29).

In summary, the combined use of receptor specific monoclonal antibodies, which act as a molecular Trojan horse, and avidin–biotin technology, enable intravenous RNAi *in vivo* with siRNA in distant sites of the body, intracranial brain cancer. The present studies used a chemical conjugate of the MAb and SA. However, antibody–avidin fusion proteins have been engineered and expressed, and show a favorable pharmacokinetic profile *in vivo* in rats in the delivery of antisense agents to brain (30).

REFERENCES

1. M. T. McManus, P. A. Sharp. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* **3**:737–747 (2002).
2. P. J. Paddison, A. A. Caudy, E. Bernstein, G. J. Hannon, D. S. Conklin. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* **16**:948–958 (2002).
3. S. Spagnou, A. D. Miller, M. Keller. Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA. *Biochemistry* **43**:13348–13356 (2004).
4. A. Jahraus, T. E. Tjelle, T. Berg, A. Habermann, B. Storrie, O. Ullrich, *et al. In vitro* fusion of phagosomes with different endocytic organelles from J774 macrophages. *J. Biol. Chem.* **273**:30379–30390 (1998).
5. W. M. Pardridge. shRNA and siRNA delivery to the brain. *Adv. Drug Deliv. Rev.***59**:141–152 (2007).
6. Y. Zhang, Y. F. Zhang, J. Bryant, A. Charles, R. J. Boado, W. M. Pardridge. Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clin. Cancer Res.* **10**:3667–3677 (2004).
7. N. M. Green. Avidin and streptavidin. *Methods Enzymol.* **184**:51–67 (1990).
8. T. Suzuki, D. Wu, F. Schlachetzki, J. Y. Li, R. J. Boado, W. M. Pardridge. Imaging endogenous gene expression in brain cancer *in vivo* with 111In-peptide nucleic acid antisense radiopharmaceuticals and brain drug-targeting technology. *J. Nucl. Med.* **45**:1766–1775 (2004).
9. W. M. Pardridge, Y. S. Kang, J. L. Buciak, J. Yang. Human insulin receptor monoclonal antibody undergoes high affinity binding to human brain capillaries *in vitro* and rapid transcytosis through the blood–brain barrier *in vivo* in the primate. *Pharm. Res.* **12**:807–816 (1995).
10. W. M. Pardridge, J. L. Buciak, P. M. Friden. Selective transport of an anti-transferrin receptor antibody through the blood–brain barrier *in vivo*. *J. Pharmacol. Exp. Ther.* **259**:66–70 (1991).
11. H. J. Lee, B. Engelhardt, J. Lesley, U. Bickel, W. M. Pardridge. Targeting rat anti-mouse transferrin receptor monoclonal antibodies through blood–brain barrier in mouse. *J. Pharmacol. Exp. Ther.* **292**:1048–1052 (2000).
12. W. M. Pardridge, R. J. Boado, Y. S. Kang. Vector-mediated delivery of a polyamide ("peptide") nucleic acid analogue through the blood–brain barrier *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **92**:5592–5596 (1995).
13. D. Wu, J. Yang, W. M. Pardridge. Drug targeting of a peptide radiopharmaceutical through the primate blood–brain barrier *In vivo* with a monoclonal antibody to the human insulin receptor. *J. Clin. Invest.* **100**:1804–1812 (1997).
14. R. J. Boado, W. M. Pardridge. Ten nucleotide cis element in the 3-untranslated region of the GLUT1 glucose transporter mRNA increases gene expression via mRNA stabilization. *Brain Res. Mol. Brain Res.* **59**:109–113 (1998).
15. Y. Zhang, R. J. Boado, W. M. Pardridge. *In vivo* knockdown of gene expression in brain cancer with intravenous RNAi in adult rats. *J. Gene Med.* **5**:1039–1045 (2003).
16. S. M. Elbashir, J. Harborth, K. Weber, T. Tuschl. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* **26**:199–213 (2002).
17. R. F. Barth. Rat brain tumor models in experimental neuro-oncology: the 9L, C6, T9, F98, RG2 (D74), RT-2 and CNS-1 gliomas. *J. Neurooncol.* **36**:91–102 (1998).
18. Y. L. Chiu, T. M. Rana. RNAi in human cells: basic structural and functional features of small interfering RNA. *Mol. Cell* **10**:549–561 (2002).
19. A. de Fougerolles, M. Manoharan, R. Meyers, H. P. Vornlocher. RNA interference *in vivo*: toward synthetic small inhibitory RNA-based therapeutics. *Methods Enzymol.* **392**:278–296 (2005).
20. I. Pilz, E. Schwarz, W. Durchschein, A. Licht, M. Sela. Effect of cleaving interchain disulfide bridges on the radius of gyration and maximum length of anti-poly(D-alanyl) antibodies before and after reaction with tetraalanine hapten. *Proc. Natl. Acad. Sci. USA.* **77**:117–121 (1980).
21. M. Grzelinski, B. Urban-Klein, T. Martens, K. Lamszus, U. Bakowsky, S. Hobel, F. Czubayko, A. Aigner. RNA interference-mediated gene silencing of pleiotrophin though polyethylenimine-complexed small interfering RNAs *in vivo* exerts antitumoral effects in glioblastoma xenografts. *Hum. Gene Ther.* **17**:751–766 (2006).
22. Y. K. Song, F. Liu, S. Chu, D. Liu. Characterization of cationic liposome-mediated gene transfer *in vivo* by intravenous administration. *Hum. Gene Ther.* **8**:1585–1594 (1997).
23. K. Miyawaki-Shimizu, D. Predescu, J. Shimizu, M. Broman, S. Predescu, A. B. Malik. siRNA-induced caveolin-1 knockdown in mice increases lung vascular permeability via the junctional pathway*Am. J. Physiol. Lung. Cell. Mol. Physiol.* **290**:L405–L413 (2006).
24. P. Kumar, H. Wu, J. L. McBride, K.-E. Jung, M. H. Kim, B. L. Davidson, S. K. Lee, P. Shankar, N. Manjunath. Transvascular delivery of small interfering RNA to the central nervous system*Nature.***448**:39–43 (2007).
25. B. T. Hawkins, R. D. Egleton, T. P. Davis. Modulation of cerebral microvascular permeability by endothelial nicotinic acetylcholine

receptors. *Am. J. Physiol. Heart Circ. Physiol.* **289**:H212–H219 (2005).
26. H. J. Lee, Y. Zhang, C. Zhu, K. Duff, W. M. Pardridge. Imaging brain amyloid of Alzheimer disease *in vivo* in transgenic mice with an Abeta peptide radiopharmaceutical. *J. Cereb. Blood Flow Metab.* **22**:223–231 (2002).
27. S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**:494–498 (2001).
28. M. Egli, G. Minasov, V. Tereshko, P. S. Pallan, M. Teplova, G. B. Inamati, E. A. Lesnik, S. R. Owens, B. S. Ross, T. P. Prakash, M. Manoharan. Probing the influence of stereoelectronic effects on the biophysical properties of oligonucleotides: comprehensive analysis of the RNA affinity, nuclease resistance, and crystal structure of ten 2-*O*-ribonucleic acid modifications. *Biochemistry* **44**:9045–9057 (2005).
29. T. Yoshikawa, W. M. Pardridge. Biotin delivery to brain with a covalent conjugate of avidin and a monoclonal antibody to the transferrin receptor*J. Pharmacol. Exp. Ther.* **263**:897–903 (1994).
30. M. L. Penichet, Y. S. Kang, W. M. Pardridge, S. L. Morrison, S.U. Shin. An antibody-avidin fusion protein specific for the transferrin receptor serves as a delivery vehicle for effective brain targeting: initial applications in anti-HIV antisense drug delivery to the brain. *J. Immunol.* **163**:4421–4426 (1999).