molecular pharmaceutics

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Antibody-Mediated Targeting of siRNA via the Human Insulin Receptor Using Avidin−Biotin Technology

Chun-Fang Xia,[†] Ruben J. Boado,[†,‡] and William M. Pardridge*,[†]

Department of Medicine, UCLA, Los Angeles, California 90024, and ArmaGen Technologies, Inc., Santa Monica, California

Received October 7, 2008; Revised Manuscript Received November 17, 2008; Accepted November 25, 2008

Abstract: Delivery of short interfering RNA (siRNA) to cells in culture, and in vivo, is possible with combined use of a receptor-specific monoclonal antibody (mAb) and avidin−biotin technology. In the present studies, the luciferase gene is transiently expressed in human 293 epithelial cells. The siRNA delivery system is composed of the siRNA, monobiotinylated on the 3′-terminus of the sense strand, and a conjugate of streptavidin (SA) and a mAb to the human insulin receptor (HIR). Exposure of cells to 3′-biotinyl-siRNA bound to the HIRMAb/SA conjugate, but not to unconjugated SA, avidin, or the HIRMAb, causes a >90% reduction in luciferase gene expression. The receptor-targeted siRNA effect is maximal at 48 h after delivery of the siRNA to the cells, and the effect is lost by 7 days after a single application of the targeted siRNA in culture. The KI of the receptor-targeted siRNA inhibition of gene expresssion is 30.5 ± 11.7 nM, and significant inhibition is observed with siRNA concentrations as low as 3 nM. In conclusion, the combination of a receptor-specific targeting ligand, such as the HIRMAb, and avidin−biotin technology allows for high affinity capture of the monobiotinylated siRNA by the targeting mAb. The siRNA is effectively delivered to the cytosol of cells, and knockdown of gene expression with the HIRMAb/SA delivery system is comparable to RNA interference effects obtained with cationic polyplexes. Whereas the use of cationic polyplexes in vivo is problematic, the bond between the targeting mAb and the siRNA is stable with avidin−biotin technology, and RNAi effects at distant sites such as brain are observed in vivo following an intravenous administration of the targeted siRNA.

Keywords: Blood−brain barrier; insulin receptor; luciferase; RNAi; siRNA

Introduction

The development of therapeutics composed of short interfering RNA (siRNA) is limited by the trans-cellular delivery of these charged, large molecule drugs. In the case of brain, delivery is particularly challenging, because the siRNA must be targeted not only across the brain cell membrane but also across the brain capillary endothelial membranes, which form the blood−brain barrier (BBB) in vivo. Large molecule drugs can be targeted across the BBB and brain cell membrane with molecular Trojan horses.[1] These are receptor-specific ligands, or peptidomimetic monoclonal antibodies (mAb), which undergo receptor-mediated transport across the BBB, and receptor-mediated endocytosis into brain cells via endogenous receptor systems. The most active molecular Trojan horse for the human BBB is a mAb against the human insulin receptor (HIR), called the HIRMAb. Both chimeric and humanized forms of the HIRMAb have been genetically engineered.[2,3]

The HIRMAb could be used to deliver siRNA to brain. However, a critical factor in siRNA therapeutics for the brain is the technology used to stably link the siRNA to the

* Correspondence to this author. Mailing address: UCLA Warren Hall 13-164, 900 Veteran Ave., Los Angeles, CA 90024. Phone: (310) 825-8858. Fax: (310) 206-5163. E-mail: wpardridge@mednet.ucla.edu.

[†] UCLA.

[‡] ArmaGen Technologies, Inc.

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10.1021/mp800194y CCC: $40.75
Published on Web 12/18/2008

HIRMAb, or other molecular Trojan horse. In the past, the targeting ligand has been conjugated to cationic proteins or polymers, which form electrostatic interactions with the anionic siRNA. The attachment of the siRNA to the targeting ligand via an intermediate cationic bridge may be problematic for in vivo delivery. To date, there is no study which demonstrates the stable association of the anionic siRNA with the cationic bridge in the circulation in vivo.

An alternative linker technology, which can provide a stable linkage between the siRNA and the targeting antibody, is avidin−biotin technology.[4] In this approach, a genetically engineered fusion protein of the Trojan horse and avidin is formulated in a first vial. In parallel, the monobiotinylated drug, e.g. siRNA, is formulated in a second vial. The two vials are mixed just prior to intravenous administration. Owing to the very high affinity of avidin binding of biotin ($K_D = 10^{-15}$ M, dissociation $t_{1/2} = 89$ days),[5] there is rapid and high affinity capture of the biotinylated ligand by the avidin. Moreover, the avidin−biotin linkage between the therapeutic and the targeting antibody has been demonstrated to be stable in the blood in vivo in rats and mice for both peptides[6−8] and antisense agents, such as peptide nucleic acids.[9] With regard to the use of avidin−biotin technology for siRNA delivery, prior work demonstrated that siRNAs could be biotinylated on either the 3′ or 5′ end of the sense strand of the duplex, and conjugated to the targeting antibody, without loss of RNAi activity.[10] The purpose of the present study is to examine further the characteristics of gene knockdown with biotinylated siRNAs conjugated to an antibody delivery system. These studies examine the dose−response and time−response relationships of the siRNA pharmacologic effect in human 293 epithelial cells transiently transfected with the luciferase gene.

Methods and Materials

Materials. The sense and antisense strands were purchased separately from Dharmacon (Lafayette, CO), and annealed as described previously.[10] The sequences of the sense and antisense strands have been described previously,[10] and are 21-mers, which include two 3′-deoxythymidine residues on both strands, and a biotin residue on the 3′-terminus of the sense strand. The siRNA targets the luciferase mRNA.[11] A tetra-ethyleneglycol spacer is placed between the 3′-terminus and the biotin group.[10] [3H]-Biotin, 44 μCi/nmol, was purchased from Perkin-Elmer (Boston, MA). The human 293 cells and the Eagle MEM medium were obtained from ATCC (Rockville, MD). Lipofectamine 2000, DMEM, and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Biocoat collagen type I coated 24-well cluster dishes were purchased from BD Biosciences (Bedford, MA). Recombinant streptavidin (SA) and all other reagents were obtained from Sigma (St. Louis, MO).

Production of HIRMAb Delivery System. The murine 83-14 HIRMAb was purified by protein G affinity chromatography from ascites generated in BALB/C mice. Recombinant SA was conjugated to the HIRMAb with a stable thioether linkage in a 1:1 molar ratio, and purified with a 2.6 × 95 cm column of Sephacryl S-300HR gel filtration chromatography as described previously.[12] The separation of the HIRMAb/SA conjugate, and unconjugated SA, was monitored by measurement of the elution profile off the column of 3H-biotin, which was added to the sample prior to addition to the column.

Biotin Delivery to Human 293 Cells. The biological activity of the HIRMAb/SA conjugate as a delivery system in 293 cells had not been tested previously. Therefore, the cellular uptake of 3H-biotin, bound to either SA alone or the HIRMAb/SA conjugate was measured. The 293 cells (400,000 cells/well) were plated in collagen coated 24-well cluster dishes in DMEM/10% FBS. When the cells were 80% confluent, the medium was aspirated, and the wells washed with 0.01 M Tris/0.15 M NaCl/pH = 7.4 (TBS), and the

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incubation was initiated by the addition of 400 μL of TBS containing 0.5 μCi/mL of 3H-biotin, and 10 μg/mL of either SA or the HIRMAb/SA conjugate. Uptake was terminated at 2, 10, 30, 60, and 120 min of incubation at 37C. The medium was aspirated, and the wells were washed extensively with ice cold TBS. The monolayer was solubilized in 0.5 mL/well of 1 N NaOH, and the sample 3H radioactivity was determined in a liquid scintillation counter, and the sample protein was determined with the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL). The cell volume of distribution (VD), in units of μL/mg protein, was determined from the ratio of DPM/mg protein in the dish, divided by the DPM/μL of medium.

Luciferase Transfection of 293 Cells. The luciferase expression plasmid is clone 790, which was described previously,[13] and is a pCEP4 vector backbone containing the luciferase cDNA originating from the pGL2 plasmid. The clone 790 plasmid DNA was produced with the EndoFree Maxiprep kit from Qiagen (Valencia, CA). The 293 cells (400,000 cells/well) were plated in collagen coated 24-well cluster dishes in DMEM/10% FBS. When the cells were 80% confluent, the medium was aspirated, and the wells washed with serum free DMEM. For transfection, the plasmid DNA and Lipofectamine 2000 were separately mixed in DMEM, and then pooled in a larger volume of DMEM, and 0.4 mL was plated per well, which contained 2.0 μL/well of Lipofectamine 2000, 0.8 μg/well of clone 790 plasmid DNA, followed by incubation at 37C for 4 h. The medium was then aspirated, and the wells washed with fresh MEM containing 1% FBS, followed by a 1 h incubation to remove any residual Lipofectamine 2000 from the dishes. This medium was then aspirated, and the wells were washed with serum free MEM, prior to initiation of 24−48 h exposures of the cells to antiluciferase siRNA.

siRNA Incubations of Transfected Cells. The biotinylated siRNA was added to each well in a volume of 0.4 mL/well of MEM medium containing 0 to 115 nM siRNA, and 0 to 400 nM HIRMAb/SA, unconjugated SA, unconjugated HIRMAb, or avidin. At the end of the incubation, the medium was aspirated, and the monolayer was lysed in luciferase lysis buffer (Promega, Madison, WI) for determination of BCA protein, and luciferase enzyme activity using a Berthold luminometer, as described previously.[10] The relative light units (RLU) were converted to pg of luciferase, based on a standard curve, and the data were expressed as pg luciferase/mg protein.

Statistical Evaluations. The half-inhibition constant, KI, of targeted siRNA knockdown of luciferase gene expression was determined by nonlinear regression analysis using the PAR subroutine of the BMDP Statistical Software package. Statistical significance was judged to be at the $p < 0.05$ level and was determined with either analysis of variance (ANOVA) or Student's *t* test.

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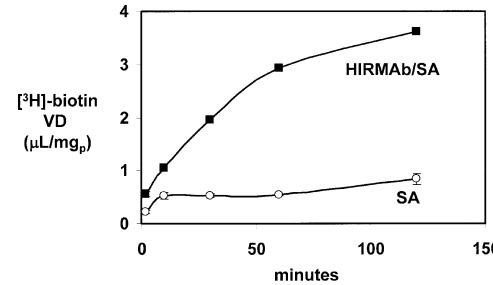

Figure 1. Time course of uptake by 293 cells of 3H-biotin bound to either streptavidin (SA) or a conjugate of SA and the HIRMAb, designated HIRMAb/SA. The concentration in the medium of the 3H-biotin, the SA, or the HIRMAb/SA was 11 nM, 200 nM, and 50 nM, respectively. Data are mean ± SE ($n = 4$ dishes per point).

Table 1. Luciferase Gene Knockdown with 3′-Biotinyl siRNA Targeted with the HIRMAb/SA Delivery System[a]

	luciferase activity (pg/mg protein)	
treatment	24 h	48 h
control	402 ± 8	1452 ± 10
biotinyl siRNA	370 ± 16	1347 ± 57
biotinyl siRNA plus SA	394 ± 30	1281 ± 15
biotinyl siRNA plus HIRMAb/SA	73 ± 6[b]	97 ± 29[b]

[a] Mean ± SE ($n = 4$ dishes/point). The medium concentration of biotinyl siRNA, the SA, and the HIRMAb/SA was 115 nM, 200 nM, and 400 nM, respectively. [b] $p < 0.01$ difference from control (ANOVA).

Results

Human 293 cells have not been previously used as a model for drug targeting with the HIRMAb. Therefore, the initial study evaluated the uptake of 3H-biotin by the human 293 cells in the presence of either the HIRMAb/SA conjugate or unconjugated SA. This study shows there is enhanced uptake of biotin following capture of the biotin by the HIRMAb/SA conjugate, as compared to SA alone (Figure 1). These results demonstrate that the HIR is expressed on the 293 cells, and that the HIRMAb is active as a delivery system in this cell.

In order to confirm that high levels of transgene expression could be observed in 293 cells following 48 h incubations in serum free medium, the level of luciferase expression was measured at in 293 cells incubated with 0, 1, or 10% fetal bovine serum (FBS) for 48 h after lipofection; the level of luciferase enzyme activity was 699 ± 42, 1484 ± 48, and 1384 ± 24 pg luciferase/mg protein (mean ± SE, $n = 4$ dishes). Since high levels of luciferase expression were observed without the addition of serum, all subsequent studies were performed in serum free medium for 24−48 h periods following lipofection.

Luciferase transgene expression in 293 cells increased with the duration of the incubation following the 4 h transfection. The level of luciferase expression in untreated cells was 402 ± 8 and 1452 ± 10 pg of luciferase/mg of protein after 24 and 48 h of incubation, respectively (Table 1). The addition

Table 2. Lack of RNAi by Protein Delivery System without siRNA[a]

treatment	luciferase activity (pg/mg protein)
control	583 ± 37
HIRMAb/SA	525 ± 32
SA	674 ± 27
HIRMAb	603 ± 24
avidin	574 ± 12

[a] Mean ± SE (n = 4 dishes/point). All measurements made at 24 h after treatment. All proteins added at 150 nM. No significant differences from control by ANOVA.

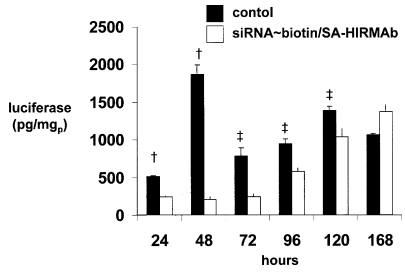

Figure 2. Luciferase gene expression in 293 cells at 1, 2, 3, 4, 5, and 7 days of incubation, following a 3 h lipofection with the luciferase expression plasmid. After lipofection, the plates were washed, and new medium was added containing either 0 or 115 nM 3′-biotinyl siRNA conjugated to HIRMAb/SA. The effect of the targeted siRNA diminished with time and was lost by 7 days of incubation. Data are mean ± SE (n = 4 dishes per point). † $p < 0.001$ difference from control; ‡ $p < 0.05$ difference from control, as determined by ANOVA.

to the medium of the 3′-biotinyl siRNA alone, or 3′-biotinyl siRNA conjugated to SA, had no effect on luciferase gene expression at 24 or 48 h (Table 1). However, the addition to the medium of the 3′-biotinyl siRNA conjugated to the HIRMAb/SA delivery system caused a 82% and 93% inhibition of luciferase gene expression at 24 and 48 h, respectively (Table 1). The addition of the HIRMAb/SA, per se, to the medium did not cause the inhibition of luciferase gene expression. As shown in Table 2, the addition of the HIRMAb/SA, without siRNA, had no effect on luciferase gene expression. Similarly, the addition of unconjugated SA, unconjugated avidin, or unconjugated HIRMAb had no effect on luciferase gene expression (Table 2).

The time course of luciferase gene expression in the 293 cells, with or without HIRMAb-mediated siRNA delivery, is shown in Figure 2. In the absence of siRNA, luciferase gene expression in the 293 cells peaked at 48 h after the 3 h lipofection, and persisted for at least 7 days in culture. The knockdown of luciferase gene expression caused by the single addition of the 3′-biotinyl siRNA, conjugated to the HIRMAb/SA, was significant for 5 days, and lost by 7 days in culture. The % inhibition in luciferase gene expression at 1, 2, 3, 4, and 5 days of incubation is 52% ($p < 0.001$), 89% ($p < 0.001$), 69% ($p < 0.05$), 38% ($p < 0.05$), and 26% ($p < 0.05$), respectively.

The dose response of knockdown of luciferase gene expression by the 3′-biotinyl siRNA, conjugated to HIRMAb/SA, is given in Figure 3. The % inhibition in luciferase gene expression at 1, 3, 10, 50, and 115 nM targeted siRNA is 7% ($p > 0.05$), 19% ($p < 0.05$), 23% ($p < 0.01$), 83% ($p < 0.001$), and 97% ($p < 0.001$), respectively. The KI of the targeted siRNA was determined by nonlinear regression analysis to be 30.5 ± 11.7 nM siRNA (Figure 3).

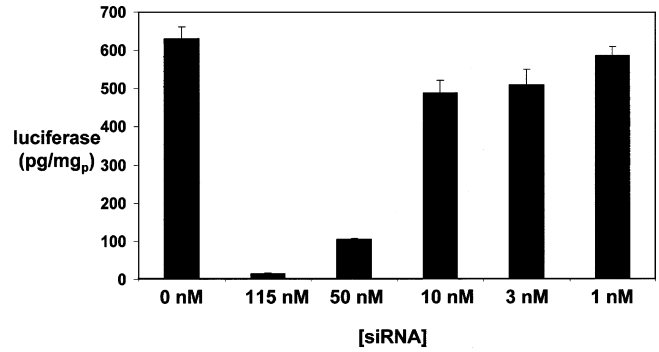

Figure 3. Luciferase gene expression in 293 cells at 48 h of incubation with varying concentrations of targeted siRNA ranging from 0 to 115 nM. The molar concentration of the HIRMAb/SA in the medium was 3-fold in excess of the respective concentration of 3′-biotinyl siRNA. Data are mean ± SE (n = 4 dishes per point).

Discussion

The results of this study are consistent with the following conclusions. First, the human 293 epithelial cell line is shown to be a useful model for receptor-mediated delivery of siRNA, and expresses a functional HIR that mediates the uptake of biotinylated ligands conjugated to the HIR (Figure 1). Second, the combination of targeting the HIR with a receptor-specific mAb, the biotinylated siRNA, and the streptavidin bridge stably linking the siRNA to the mAb, leads to a >90% inhibition of target gene expression (Table 1). Third, only the combined use of the receptor-specific mAb and avidin−biotin technology leads to RNA interference, as unconjugated SA, HIRMAb, or avidin has no effect on gene expression (Tables 1 and 2). Fourth, the receptor-mediated RNAi effect persists for at least 5 days (Figure 2), is observed at siRNA concentrations as low as 3 nM (Figure 3), and the siRNA K_i is 30.5 ± 11.7 nM (Figure 3).

Previous work on the receptor targeting of siRNA to cells used the human U87 glial cell line.[10] However, for testing siRNA effects in transiently transfected cells, it is not possible to place the transfected cells in medium containing serum, since serum nucleases degrade the siRNA over prolonged incubations in culture. The combination of Lipofectamine 2000 exposure, and prolonged incubation in serum free medium, results in reduced viability of U87 cells (unpublished observations). However, high levels of luciferase expression are observed in the 293 cells incubated without serum for 48 h (Results). The level of luciferase gene expression in the 293 cells (Table 1) is nearly 10-fold greater than in U87 cells.[10] Human 293 cells express a functional insulin receptor, and the HIRMAb/SA conjugate mediates

enhanced cell uptake of biotin (Figure 1). In addition, the present studies show that the addition to the medium of high concentrations (150 nM) of streptavidin (SA), or avidin, has no effect on luciferase trans-gene expression (Table 2). Such high concentrations of SA or avidin would be expected to sequester medium biotin. Biotin depletion can affect gene expression, perhaps through biotinylation of histones.[14] Nevertheless, biotin may be generated within the cell via a biotin recycling process, owing to the ubiquitous expression of biotinidase.[14] The observation that SA or avidin has no affect on luciferase transgene expression indicates that the HIRMAb/SA delivery system does not have nonspecific affects on transgene expression (Table 2).

The knockdown in luciferase gene expression caused by siRNA targeting with the HIRMAb/SA conjugate is >90%, and the maximal effect is observed at 48 h of incubation (Figure 2). The knockdown of target gene expression does not persist beyond 5 days (Figure 2). A similar time course is observed in siRNA delivery to cells with cationic agents, where the peak effect is at 48 h,[15] and the RNAi effect is lost by 5 days.[16]

Targeted siRNA inhibits gene expression at concentrations as low as 3 nM, and exhibits a dose response relationship (Figure 3). The KI of knockdown of luciferase gene expression with the 3′-biotinyl siRNA targeted to the 293 cells with the HIRMAb/SA is 30.5 ± 11.7 nM (Results). This observation indicates the HIRMAb delivery system efficiency in cell culture is comparable to the cationic lipids, such as Oligofectamine, which produce siRNA knockdown effects with a half-inhibition constant of 50 nM siRNA.[17] However, Oligofectamine, Lipofectamine 2000, or other cationic polyplex delivery systems that work in cell culture may be less effective delivery systems in vivo. The siRNA/cationic polyplexes form small nanoparticles in low ionic medium, but aggregate in physiological saline in vitro,[18] and aggregate in the circulation in vivo.[19] In contrast, the bond between the targeting antibody and the biotinylated ligand is stable in vivo,[6–9] and is effective in siRNA delivery to distant organs, such as the brain, following intravenous administration.[10]

In conclusion, these studies provide evidence for the novel aspects of siRNA delivery to cells using a combination of a receptor-specific monoclonal antibody, and the high affinity linkage of the siRNA to the antibody using avidin−biotin technology. There are 2 approaches to the production of the mAb−avidin complex. First, the mAb may be chemically conjugated to either avidin or SA with a thio-ether linkage, such as used in this study. Second, a genetically engineered mAb−avidin fusion protein may be produced, as recently described for the HIRMAb.[4] SA, not avidin, is the preferred biotin binder when the thio-ether conjugate is prepared. Avidin is a cationic protein, whereas SA is a neutral/acidic protein. The cationic nature of avidin causes high nonspecific binding to cells via a process that is completely inhibited by other polycationic proteins, such as protamine.[20] Previous work showed there is no nonspecific binding of SA to cells,[20] and the data in Figure 1 show there is no nonspecific uptake of siRNA by cells when the siRNA is bound to SA. Following the intravenous injection of a chemical conjugate of an mAb and avidin, there is also nonspecific binding of avidin to cells in vivo, which accounts for the rapid removal of the mAb−avidin conjugate from blood.[21] However, the nonspecific binding of avidin is not observed in vivo, when the avidin monomer is part of a genetically engineered fusion protein of avidin and the mAb. The clearance from blood of a mAb−avidin fusion protein is determined by the IgG, not the avidin part of the fusion protein.[22] Recently, a HIRMAb−avidin fusion protein has been genetically engineered, expressed in CHO cells in biotin-depleted medium, and validated with biotin binding assays and insulin receptor binding assays.[4] The HIRMAb−avidin fusion protein is biologically active in humans and Old World primates, such as the Rhesus monkey, but is not active in rodents. The genetically engineered HIRMAb−avidin fusion protein is a novel delivery system for targeting biotinylated ligands, including biotinylated siRNA, to brain in humans.

Acknowledgment. This work was funded in part by NIH Grant R43-NS-062458.

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