Pharmaceutical Research, Vol. 23, No. 7, July 2006 (© 2006)
DOI: 10.1007/s11095-006-0274-x

Research Paper

Decline in Exogenous Gene Expression in Primate Brain Following Intravenous Administration Is Due to Plasmid Degradation

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Received October 20, 2005; accepted February 21, 2006

Purpose. Nonviral gene transfer to the brain of adult Rhesus monkeys is possible with a single intravenous administration of plasmid DNA that is encapsulated in the interior of pegylated immunoliposomes, which are targeted across membrane barriers *in vivo* with a monoclonal antibody to the human insulin receptor.
Methods. The present studies measure the rate of decay of luciferase gene expression in the Rhesus monkey with luciferase enzyme assays, Southern blotting, and real-time polymerase chain reaction.
Results. Luciferase enzyme activity in frontal cortex, cerebellum, and liver decays with a $t_{1/2}$ of 2.1 ± 0.1, 2.6 ± 0.2, and 1.7 ± 0.01 days, respectively. Luciferase plasmid in brain and liver was detectable by Southern blotting at 2 days, but not at 7 or 14 days. The concentration of luciferase plasmid DNA in brain and liver was measured by real-time polymerase chain reaction, and decayed with $t_{1/2}$ of 1.3 ± 0.3 and 2.7 ± 0.5 days, respectively.
Conclusions. The maximal concentration of luciferase plasmid DNA in Rhesus monkey brain was 3–4 molecules/cell following an i.v. administration of 12 μg/kg pegylated immunoliposome encapsulated plasmid DNA. These results demonstrate that the rate of loss of exogenous gene expression in the primate *in vivo* correlates with the rate of DNA degradation of the exogenous plasmid DNA.

KEY WORDS: insulin receptor; luciferase; nonviral gene transfer; real-time PCR.

INTRODUCTION

Nonviral plasmid gene therapeutics may be delivered to distant target sites *in vivo* following an intravenous administration with the use of the pegylated immunoliposome (PIL) gene targeting technology (1). A single plasmid DNA molecule is encapsulated in the interior of a 100-nm liposome. The surface of the liposome is conjugated with several thousand strands of 2,000-Da polyethyleneglycol (PEG). The tips of 1–2% of the PEG strands are conjugated with a receptor-specific targeting monoclonal antibody (MAb). In prior studies, plasmid DNA encoding luciferase or β-galactosidase was delivered to the brain of adult Rhesus monkeys with an intravenous injection of PILs targeted with an MAb to the human insulin receptor (HIR) (2). The expression of luciferase and β-galactosidase in Rhesus monkey brain was determined 2 days after a single intravenous injection. In parallel studies, luciferase gene expression was measured in the retina of Rhesus monkeys, and luciferase enzyme activity in the retina decayed with a $t_{1/2}$ of 2.0 ± 0.1 days (3). The decay in gene expression may be due either to promoter inactivation, or to plasmid degradation.

The purpose of the present studies was to measure luciferase gene expression in Rhesus monkey brain at periods lasting up to 14 days following a single intravenous injection of HIRMAb-targeted PILs. In parallel with measurements of luciferase enzyme activity in primate brain, the level of the exogenous plasmid DNA in primate brain was measured with real-time polymerase chain reaction (PCR) assays, and by Southern blotting. The correlation of the luciferase enzyme assay and the real-time PCR assay allows for a determination as to whether the loss with time of luciferase gene expression is attributable to promoter inactivation or to plasmid DNA degradation.

MATERIALS AND METHODS

Intravenous Gene Administration in Rhesus Monkeys

Three healthy 5- to 10-year-old, 5- to 6-kg female Rhesus monkeys were purchased from Covance (Alice, TX, USA). A fourth rhesus monkey was sacrificed for removal of control tissues from an uninjected primate. The animals were anesthetized with 10 mg/kg ketamine intramuscular, and 5 mL sterile HIRMAb-PIL containing 70 μg of plasmid DNA was injected into each monkey via the saphenous vein with a 18-gauge catheter. The primates were euthanized at 2, 7, or 14 days after the single PIL injection of the luciferase expression plasmid (3). The brain, liver, spleen, lung, heart, kidney, and triceps skeletal muscle were removed. PIL formulation was prepared as previously described (2). The total dose of HIRMAb that was conjugated to the PIL and administered to each monkey was 1.8 mg or 300 μg/kg of

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antibody. The injection dose of PIL encapsulated plasmid DNA was 12 μg/kg. Murine HIRMAb was purified from hybridoma generated ascites by protein G affinity chromatography. The luciferase expression plasmid is clone 790, as previously described (2,4), and is driven by the SV40 promoter. The research adhered to the provisions of "Principles of Laboratory Animal Care" (NIH Publication #85-23, revised in 1985).

Luciferase Enzyme Activity Measurements

Primate organs were homogenized in 4 vol Promega lysis buffer as previously described (2). The data are reported as pg luciferase activity per mg cell protein. Based on the standard curve, 1 pg luciferase was equivalent to 14,312 ± 2,679 relative light units (RLU), which is the mean ± SE of five assays.

Isolation of Genomic DNA

Genomic DNA was isolated from monkey brain and liver with Genomic-tip 500/G columns from Qiagen (Valencia, CA, USA) according to the manufacturer's instructions. About 350 mg monkey liver tissue or 400 mg monkey brain tissue was homogenized in 0.8 M guanidine, 0.03 M Tris/8.0, 30 mM EDTA, 5% Tween 20, 0.5% Triton X-100, and 0.2 mg/mL RNase A, followed by incubation with 1 mg/mL proteinase K at 50°C for 2 h. Genomic DNA in the homogenate was retrieved by filtration through a Qiagen Genomic tip 500/G column followed with isopropanol precipitation. The A_{260}/A_{280} ratio averaged 2.0, and the yield of genomic DNA was 500 μg. The yield of genomic DNA isolation was 70% based on parallel samples labeled with ^{32}P-DNA internal standard.

Real-Time PCR

Real-time PCR primers that hybridize within the open reading frame of the firefly luciferase gene were identified with the Beacon 2.1 software (Bio-Rad, Hercules, CA, USA), which selects primers with an annealing temperature of 58 ± 2°C and PCR products with 75–150 bp. The sequence for the forward primer is 5′-TCGAAAGAAGTCGGGGAAGC, and the sequence for the reverse primer is 5′-CCTCGGGTG TAATCAGAATAGC.

Table I. Luciferase Enzyme Activity in Rhesus Monkey Organs

	pg luciferase/mg protein		
Organ	2 days	7 days	14 days
Frontal white	3.2 ± 0.3	0.4 ± 0.03	0.020 ± 0.002
Cerebellum gray	7.0 ± 1.6	1.9 ± 0.2	0.080 ± 0.009
Cerebellum white	4.0 ± 0.4	0.4 ± 0.03	0.025 ± 0.0006
Heart	0.014 ± 0.003	0.003 ± 0.0007	<0.01
Liver	15.5 ± 0.4	2.9 ± 0.2	0.14 ± 0.023
Spleen	2.8 ± 0.4	0.6 ± 0.03	0.015 ± 0.0007
Lung	1.9 ± 0.3	0.3 ± 0.03	0.017 ± 0.002
Kidney	0.6 ± 0.1	0.05 ± 0.003	<0.01
Skeletal muscle	0.13 ± 0.02	0.011 ± 0.001	<0.01

Data are mean ± SE (n = 3 replicates from a single monkey at each time point).

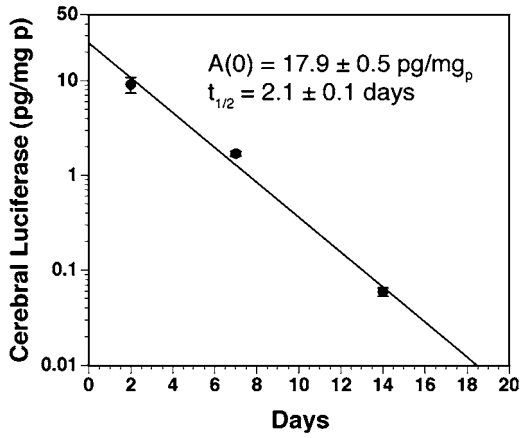

Fig. 1. Luciferase enzyme activity in Rhesus monkey frontal cortex gray matter is plotted vs. time after a single i.v. injection of HIRMAb-targeted PILs carrying clone 790 luciferase expression plasmid DNA. The y-intercept, $A(0)$, and the slope were determined by linear regression analysis. The $t_{1/2}$ of decay was determined from ln 2/slope.

Real-time PCR was performed by using Bio-Rad MBR Green Supermix and an iCycler IQ™ Real-time Detection System from Bio-Rad. For each reaction in 25 μL, 200 ng genomic DNA and 2.5 μL of 2.5 μM primer mixture were added. PCR was initiated by a 3-min incubation at 95°C, followed by 40 cycles consisting of 10 s annealing at 57.9°C, 10 s extension at 72°C, and 10 s denaturing at 95°C. Melting curves of each PCR product were determined by measuring the ratio of single-strand DNA vs. double-strand DNA at every 0.5°C temperature increase from 55 to 94°C. For quantification of luciferase DNA, the clone 790 luciferase expression plasmid was diluted into 5 ng/μL, 50 pg/μL, 500 fg/μL, 5 fg/μL, and 0.05 fg/μL aliquots containing 100 ng/μL genomic DNA isolated from normal monkey brain tissue. Real-time PCR was performed as described above, using 2 μL DNA standard solution as the template. A series of threshold cycle (C_t) values were obtained. A standard curve of luciferase DNA was generated by plotting C_t values against the logarithm of plasmid mass (fg). Plasmid mass was converted into number of plasmid molecules based on the molecular weight of the luciferase expression plasmid, 10.6 kb, and 665 Da per base pair. The standard curve was linear over 8 log orders of luciferase DNA (10^{-4} to 10^4 pg DNA).

Southern Blotting

Genomic DNA (10 μg) from brain or liver were digested with 15 U *Hin*dIII for 1 h at 37°C, resolved by gel electrophoresis in 0.8% agarose, blotted to a GeneScreen Plus membrane, and hybridized with the ^{32}P-labeled, *Hin*dIII linearized luciferase expression plasmid designated clone 734 (4). Autoradiograms were developed following exposure of Kodak Biomax film for 3 days at −70°C.

RESULTS

The luciferase enzyme activity in brain and peripheral tissues of adult Rhesus monkeys at 2, 7, and 14 days after a single intravenous injection of the HIRMAb targeted PIL is

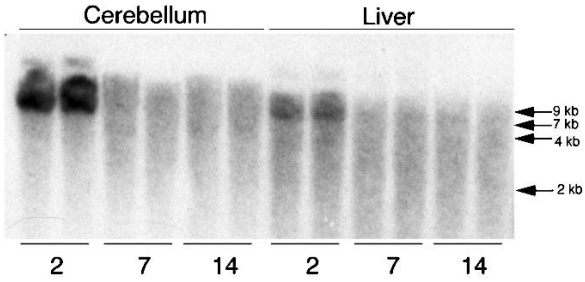

Fig. 2. Southern blot analysis of genomic DNA isolated from Rhesus monkey cerebellum and liver at 2, 7, and 14 days after a single i.v. injection of HIRMAb-targeted PILs carrying clone 790 luciferase expression plasmid DNA. The size of molecular weight standards is shown on the right side of the figure. The top of the gel is shown, indicating the sample DNA has migrated into the gel.

shown in Table I. For brain, the data in Table I include luciferase enzyme activity for frontal cortex white matter, cerebellar gray matter, and cerebellar white matter. The luciferase enzyme activity levels in frontal cortex gray matter at the 3 different days after intravenous administration are given in Fig. 1. The luciferase enzyme activity decays monoexponentially with a $t_{1/2}$ of 2.1 ± 0.1 days (Fig. 1). The luciferase data for cerebellar gray matter and for primate liver were also analyzed by a monoexponential function that yielded a $t_{1/2}$ of 2.6 ± 0.2 days for cerebellar gray matter and 1.7 ± 0.01 days for primate liver ($r = 0.99$).

Extracts of genomic DNA isolated from the primate brain 2, 7, and 14 days after administration were analyzed by Southern blot analysis. Plasmid DNA in cerebellum and liver was detectable at 2 days after i.v. administration, and barely detectable at 7 and 14 days after administration (Fig. 2). Levels of luciferase DNA in primate brain were quantitated by real-time PCR. A luciferase standard curve was established, and this standard curve was linear over 8 log orders of luciferase plasmid DNA concentration (Materials and Methods). Luciferase plasmid DNA concentrations in brain and liver were estimated from the real-time PCR C_t value (Table II) and the luciferase C_t standard curve. The concentrations of plasmid DNA in brain and liver at 2, 7, and 14 days after i.v. administration are given in Table II. The luciferase plasmid standard curve allowed for the conversion of C_t values into fg of luciferase plasmid DNA per 200 ng genomic DNA, or ng of luciferase plasmid DNA per 100 g tissue wet weight (Table II). Based on the size of the luciferase plasmid DNA, 10.6 kb, ng of plasmid DNA were converted into molecules of plasmid DNA per 200 ng genomic DNA (Table II). The luciferase plasmid concentrations in brain and liver (Table II) were analyzed by a monoexponential decay curve, and the intercept and slopes are given in Table III. These data show that the half-life of decay of luciferase plasmid DNA in primate brain and liver is 1.3 ± 0.3 and 2.7 ± 0.5 days, respectively.

Table III. Linear Regression Analysis of Luciferase Plasmid DNA Content in Rhesus Monkey Brain following IV Administration of PILs

Organ	Parameter	Value
Brain	$A(0)$	798 ± 123 fg luciferase DNA/200 ng DNA
	k	0.55 ± 0.11 days^{-1}
	$t_{1/2}$	1.3 ± 0.3 days
Liver	$A(0)$	248 ± 18 fg luciferase DNA/200 ng DNA
	k	0.26 ± 0.04 days^{-1}
	$t_{1/2}$	2.7 ± 0.5 days

Parameters were computed from the data in Table II by linear regression analysis. $A(0)$ and k are the y-intercept and slope, respectively. The $t_{1/2}$ was computed from ln 2/k. The correlation coefficients of the linear regression analysis were 0.98 and 0.99 for brain and liver, respectively. Tissue analyzed is frontal brain matter.

DISCUSSION

The results of these studies are consistent with the following conclusions. First, luciferase gene expression in primate brain and liver decays with a $t_{1/2}$ of about 2 days following a single i.v. injection of the luciferase expression plasmid encapsulated inside PILs targeted to brain and liver with an HIRMAb. Second, the cause of the transient duration of luciferase gene expression is plasmid degradation, because the $t_{1/2}$ of decay of luciferase plasmid DNA concentration in brain and liver, 1–2 days (Table III), approximates the $t_{1/2}$ of decay of luciferase enzyme activity (Fig. 1).

Episomal plasmid DNA gene expression is generally transitory in cultured cells *in vitro* or in organs *in vivo*, where the vector DNA is not permanently integrated into the host genome. One cause for loss of exogenous gene expression is gene silencing without loss of vector DNA (6), owing to promoter inactivation (7). Another potential cause of loss of gene expression is vector DNA degradation. Plasmid DNA is unstable in the cytosol owing to cellular DNase (8), and is degraded with a $t_{1/2}$ of 50–90 min following DNA microin-

Table II. Quantitation of Luciferase cDNA in Rhesus Monkey Brain and Liver by Real-Time PCR

Organ	Days	C_t	luc DNA (fg/200 ng DNA)	luc DNA (ng/100 g tissue)	luc DNA (molecules/200 ng DNA)
Brain	2	22.5 ± 0.1	417 ± 45	284 ± 31	34,528 ± 3,784
	7	27.7 ± 0.1	7.6 ± 0.7	5.2 ± 0.5	631 ± 60
	14	31.3 ± 0.2	0.48 ± 0.07	0.33 ± 0.05	40 ± 6
Liver	2	23.6 ± 0.08	177 ± 11	120 ± 8	14,656 ± 944
	7	25.9 ± 0.08	30.6 ± 1.8	21 ± 1	2,534 ± 147
	14	27.7 ± 0.07	7.8 ± 0.4	5.3 ± 0.3	642 ± 32

Real-time PCR C_t values were converted into fg luciferase (luc) plasmid DNA per 200 ng genomic DNA with the luciferase standard curve (Methods). The mass of luciferase plasmid DNA was converted into molecules of plasmid DNA with the molecular size, 10.6 kb, of the luciferase expression plasmid. Data are mean ± SD (n = 3–4). Tissue analyzed is frontal brain matter.

jection into the cell (9). Unlike chromosomal DNA, which has a highly ordered chromatin structure that makes the DNA inaccessible to DNase (10), plasmid DNA lacks a higher-order chromatin structure, and is particularly vulnerable to cellular endonucleases, at "hot spots," or DNase-hypersensitive sites (11). The present studies show that the basis for the transitory expression of the luciferase expression plasmid in primate brain and liver *in vivo* following intravenous administration and delivery with PILs is plasmid degradation. Southern blot (Fig. 2) shows the loss of measurable luciferase plasmid DNA, and rate of plasmid DNA degradation is quantitated with real-time PCR (Table II). The luciferase transcriptional unit incorporated in the plasmid DNA is composed of the SV40 promoter, the luciferase cDNA, followed by SV40 3′-untranslated region (UTR). The luciferase expression plasmid used in these studies lacks any chromosomal derived gene sequences that can interact with the nuclear matrix, such as a matrix attachment region (MAR). Such sequences allow plasmid DNA to attach to the nuclear matrix (12), which may inhibit the access of cellular nuclease to the exogenous DNA.

Real-time PCR allows for quantitation of the concentration of luciferase plasmid DNA in brain and liver. The level of luciferase gene expression in cerebellum and cortex is comparable (Table I), which parallels previous studies on the level of β-galactosidase gene expression in Rhesus monkey (2). The peak luciferase enzyme activity in liver is about 2-fold greater than in brain (Table I). However, the peak luciferase plasmid DNA concentration in brain is about 3-fold greater than in liver (Table III), and this reflects organ differences in genomic DNA. The luciferase plasmid DNA concentration in brain and liver is expressed relative to the amount of genomic DNA (Tables II and III). The concentration of genomic DNA in liver is about 3-fold greater than the concentration of genomic DNA in brain (13). The earliest time point of analysis of gene expression in these studies was 2 days. Prior work on β-galactosidase gene expression shows the exogenous gene does not reach maximal expression until 2 days after injection, and is submaximal at 1 day after administration (14). The time course of luciferase gene expression is believed to parallel the time course of β-galactosidase gene expression, because the bacterial β-galactosidase enzyme and the luciferase enzyme are both rapidly degraded *in vivo*. The half-time of the luciferase enzyme in cells is about 3 h (15). Although the half-time of the bacterial β-galactosidase protein in cultured cells is about 20 h (16), this protein is degraded much faster *in vivo* in animals. Bacterial β-galactosidase is completely degraded within 4 h after administration in mice (17,18). Therefore, both the luciferase and bacterial β-galactosidase proteins have short half-times *in vivo*, which means the duration of expression of enzyme activity parallels the persistence of the transgene.

Estimates are made on the number of luciferase plasmid DNA molecules in brain per 200 ng of genomic DNA, based on the molecular size of the plasmid DNA. These data are given in Table II for both brain and liver at 2, 7, and 14 days after intravenous administration of the exogenous gene. Results of a linear regression analysis of the luciferase plasmid levels are shown in Table III, which gives $A(0)$, the maximal mass of luciferase plasmid DNA in brain or liver after an i.v. injection. The level of luciferase DNA in brain and liver decays with a half-time of 1.3 ± 0.3 and 2.7 ± 0.5 days, respectively (Table III). Therefore, the half-time of decay of luciferase enzyme activity (Fig. 1) closely parallels the half-time of plasmid DNA in monkey brain or liver *in vivo* (Table III). This observation suggests that the luciferase enzyme is rapidly degraded *in vivo*, such that the limiting factor controlling the level of luciferase enzyme activity is the concentration of luciferase plasmid DNA. This observation corroborates other work showing the half-time of luciferase enzyme activity *in vivo* is only 2–3 h (15).

The $A(0)$ for primate brain is 798 ± 123 fg luciferase plasmid DNA per 200 ng of genomic DNA (Table III). Based on the molecular size of the luciferase expression plasmid, 10.6 kb (19), the $A(0)$ corresponds to 65,342 ± 1,563 molecules per 200 ng genomic DNA. Assuming there are 10 pg of genomic DNA per cell (13,20), these calculations indicate the maximum number of luciferase plasmid DNA molecules per primate brain cell is 3.3. These calculations are consistent with other studies in rats showing that pharmacologic effects in brain, e.g., experimental Parkinson's disease, are achieved with the delivery of 5–10 plasmid DNA molecules per brain cell (21). Therefore, therapeutic levels of plasmid DNA in brain cells of the adult Rhesus monkey are achieved with an intravenous dose of PIL-encapuslated plasmid DNA of 70 μg (Materials and Methods). The level of luciferase plasmid DNA at 2 days after injection is 0.28 μg per 100 g brain (Table II). Because the Rhesus monkey brain weighs 100 g (5), the brain uptake of the plasmid DNA is 0.4% of the 70 μg injected dose at 2 days after i.v. administration. Therefore, the uptake of PIL encapsulated plasmid DNA approximates the measured peak uptake of the nonliposome conjugated HIRMAb at 3 h after an i.v. injection, which is 2% of the injected dose per Rhesus monkey brain (5).

In summary, 3–4 plasmid DNA molecules are delivered per brain cell following the i.v. injection of PIL encapsulated plasmid DNA, at a dose of 70 μg DNA per 6 kg animal or 12 μg/kg in the adult Rhesus monkey. The exogenous gene expression decays with time in parallel with the degradation of the exogenous plasmid DNA. It is possible that the persistence of plasmid DNA in the brain of higher animals may be sustained for longer periods with reformulations of the plasmid DNA that render the vector less sensitive to cellular nuclease activity.

ACKNOWLEDGMENTS

This work was supported by a grant from the Neurotoxin Exposure Treatment Research Program of the U.S. Department of Defense, and by NIH grant NS-53540.

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