Organ-specific gene expression in the rhesus monkey eye following intravenous non-viral gene transfer

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Purpose: The transfer of exogenous genes to the entire retina and other ocular structures is possible with a vascular route of gene delivery using a non-viral gene transfer method. The present studies examine the extent to which either β-galactosidase or luciferase expression plasmids are targeted to the retina in the adult rhesus monkey following intravenous administration. In addition, these studies examine the pattern of organ expression of the transgene in the rhesus monkey depending on whether the plasmid is under the influence of a widely expressed promoter, the SV40 promoter, or an ocular-specific promoter, the opsin promoter.

Methods: The plasmid DNA with either the SV40 or opsin promoter is encapsulated in the interior of pegylated immunoliposomes (PILs), which are targeted across the blood-retinal barrier and into ocular cells with a monoclonal antibody to the human insulin receptor. Following a single intravenous injection of the PIL carrying the transgene, the animals were sacrificed 2, 7, or 14 days later for the measurement of β-galactosidase or luciferase gene expression in the monkey eye and peripheral organs.

Results: Histochemistry showed expression of the β-galactosidase gene throughout the entire primate retina including the photoreceptor cells with either an SV40 or a bovine opsin promoter. Whereas the SV40 promoter enables gene expression in other organs of the primate (brain, liver, spleen), the opsin promoter restricted trans-gene expression to the primate eye, as there was no gene expressed in other organs. The retinal luciferase activity at 2 days after administration was 9.6±0.4 pg luciferase/mg protein, and at 14 days after administration was still comparable to maximal levels of luciferase gene expression in the mouse or rat. Confocal microscopy with antibodies to the insulin receptor and to β-galactosidase demonstrated co-localization in the retina, with high expression of the trans-gene and the insulin receptor in the inner segments of the photoreceptor cells.

Conclusions: The PIL non-viral gene transfer technology makes possible adult transgenics in 24 h. Ectopic expression of exogenous genes in organs other than the target organ is made possible with the use of organ specific promoters, and gene expression in the primate is restricted to the eye when the trans-gene is under the influence of the opsin promoter. Plasmid-based gene expression is still in the therapeutic range for 2-3 weeks after a single intravenous administration. Exogenous genes are expressed throughout the entire primate retina following the delivery of the gene to the eye via a trans-vascular route.

Many forms of blindness are potentially treatable with retinal gene therapy [1,2]. The retinal photoreceptor cells can be transduced with sub-retinal injections of viruses such as adeno-associated virus (AAV) [3]. The portion of the human retina that is transduced with a sub-retinal injection is localized to the injection site [4,5]. Conversely, global expression of an exogenous gene throughout the human retina may be possible with a transvascular route to the eye following intravenous administration. However, viral vectors do not cross the blood-retinal barrier (BRB). In mice a non-viral gene delivery system comprised of pegylated immunoliposomes (PILs) can be targeted across the BRB following intravenous administration, and this leads to expression of an exogenous gene in the eye [6]. In this approach, the therapeutic gene is incorporated in non-viral plasmid DNA, which is encapsulated in the interior of 85 nm anionic liposomes [7,8]. The surface of the liposome is conjugated with several thousand strands of polyethylene glycol (PEG), which stabilizes the liposome in the circulation, minimizes liposome uptake by the reticuloendothelial system, and enables a sustained circulation time of the liposome in the blood. The tips of 1-2% of the PEG strands are conjugated with a targeting ligand such as a peptidomimetic monoclonal antibody (MAb). The targeting ligand binds to specific receptors expressed at both the BRB and the plasma membrane of retina and ocular cells to trigger receptor-mediated endocytosis across the BRB and receptor-mediated endocytosis into cells of the eye [6].

Exogenous genes have been targeted to the mouse eye and retinal pigmented epithelium (RPE) with PILs formulated with a MAb to the mouse transferrin receptor (TfR) [6]. This resulted in widespread expression of the exogenous gene throughout the RPE, but there was no measureable gene expression in the photoreceptor cells of the mouse retina [6]. The absence of gene expression in the photoreceptor layer is attributed to the minimal expression of TfR on the plasma membranes in the outer nuclear layer (ONL) of the retina [9,10]. The reduced expression of the TfR in the ONL is con-
sistent with very low concentrations of iron and ferritin in this region of the retina [10]. Conversely, the cells of the ONL express high levels of the insulin receptor (IR) [11,12] and a targeting ligand that accesses the IR could deliver genes to the ONL. The 83-14 murine MAb to the human insulin receptor (HIR) is rapidly transported across the blood-brain barrier (BBB) of Old World primates, owing to high expression of the IR on the primate BBB [13]. Gene transfer with the HIRMAb-targeted PIL enables the global delivery of exogenous genes to the brain of the rhesus monkey following intravenous injection [14]. The IR is also expressed at the BRB [11,12]. Therefore, the present studies determine the extent to which endogenous genes are expressed in the Rhesus monkey retina following a single intravenous injection of non-viral plasmid DNA encapsulated in a PIL that is targeted to the HIR. Owing to the genetic similarity between humans and Old World monkeys such as the Rhesus monkey [15], the 83-14 HIRMAb cross-reacts with the Rhesus monkey IR [13]. In the present studies, plasmids encoding either β-galactosidase or luciferase are administered by peripheral venous injection, and gene expression in the primate retina is measured with the luciferase assay, β-galactosidase histochemistry, and confocal microscopy using antibodies directed against β-galactosidase and the HIR. The β-galactosidase expression plasmid, under the influence of the SV40 promoter, is alternatively designated pSV-β-galactosidase or clone 756, as described previously [14]. The luciferase expression plasmid, under the influence of the SV40 promoter, was derived from the pCEP4 plasmid, and is designated clone 790 as described previously [14].

A second goal of the present work was to examine the organ specificity of gene expression with the PIL gene transfer approach. If the exogenous gene is under the influence of a widely expressed promoter, such as the SV40 promoter, then the gene is expressed in multiple organs of the primate, including brain, liver, and spleen, following the intravenous injection of HIRMAb-targeted PILs [14]. In the present study, a β-galactosidase expression plasmid under the influence of the bovine opsin promoter, is injected into the primate following encapsulation in HIRMAb-targeted PILs. The opsin-β-galactosidase plasmid is designated pLacF, as described by Zack et al [16].

**METHODS**

**Materials:** POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and DDAB (didodecyldimethylammonium bromide) were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL), and diestearylphosphatidylethanolamine (DSPE)-PEG 2000 was obtained from Shearwater Polymers (Huntsville, AL). DSPE-PEG 2000-maleimide was custom synthesized by Shearwater Polymers. [α-32P]dCTP (3000 Ci/mm mol) was from NEN Life Science Products Inc. (Boston, MA). The nick translation system was from Invitrogen (San Diego, CA). 5-Bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), IGEPAL CA-630 and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The 2-iminothiolane (Traut’s reagent) and bicinechinonic acid (BCA) protein assay reagents were obtained from Pierce Chemical Co. (Rockford, IL). The 83-14 murine MAb to the HIR was purified by protein G affinity chromatography from hybridoma generated ascites. The luciferase expression plasmid is designated clone 790 and is derived from the pCEP4 plasmid under the control of the SV40 promoter as described previously [17]. The pSV-β-galactosidase expression plasmid driven by the SV40 promoter is designated clone 756 as described previously [7]. The pLacF expression plasmid under the influence of the bovine rhodopsin promoter was provided by Dr. Don Zack of Johns Hopkins University, and was described previously [16], and is designated clone 933. This 8 kb plasmid includes nucleotides -2174 to +70 of the bovine rhodopsin gene. The presence of this portion of the bovine rhodopsin promoter within the plasmid was confirmed by DNA sequencing using a M13 reverse sequencing primer followed by custom sequencing primers. Digestion of the rhodopsin/β-galactosidase plasmid with BamHI released the 3.0 kb insert from the 5.0 kb vector backbone.

**Pegylated immunoliposome synthesis:** Pegylated immunoliposomes (PILs) were synthesized from a total of 20 µmol of lipids, including 18.6 µmol of POPC, 0.6 µmol of DDAB, 0.6 µmol of DSPE-PEG, and 0.2 µmol of DSPE-PEG-maleimide [7,8]. Clone 756 plasmid DNA, clone 790 plasmid DNA, or pLacF plasmid DNA was produced by Maxiprep (Qiagen; Chatsworth, CA), and the supercoiled plasmid DNA (200 µg) and 1 µCi of 32P-nick translated plasmid DNA were encapsulated in the pegylated liposomes by serial extrusion through filters of 400, 200, and 100 nm pore size, which forms liposomes of 85 nm diameter [18]. The exteriorized DNA was quantitatively removed by exhaustive nuclease digestion, as described previously [18]. The 83-14 MAb containing a trace amount of 3H-labeled antibody, was thioclated with Traut’s agent and the thioclated MAb was conjugated to the pegylated liposome overnight at room temperature as described previously [7,19]. The unconjugated MAb, and the degraded exteriorized DNA were separated from the DNA encapsulated within the PIL by elution through a 1.6x18 cm column of Sepharose CL-4B in 0.05 M Hepes, pH 7.0, as described previously [7,19]. The average number of MAb molecules conjugated per liposome was 43±2 (mean±SE, n=3 syntheses). The final percentage entrapment of 200 µg of plasmid DNA in the liposome preparation was computed from the 32P radioactivity and was 35±7.5% (mean±SE, n=3 syntheses). The PIL conjugated with the HIRMAb is designated HIRMAb-PIL. The HIRMAb-PIL carrying either plasmid DNA was sterilized before injection into the primate with a 0.22 µm filter (Millipore Co., Bedford, MA) as described previously [19].

**Intravenous gene administration in Rhesus monkeys:** Three healthy 5-10 year old, 5-6 kg female Rhesus monkeys were purchased from Covance (Alice, TX) and used in this study. In addition, a fourth rhesus monkey was sacrificed for removal of control tissues from an uninjected primate. Animal care guidelines were comparable to those published by the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) and the US Public Health
Service (Public Health Service Policy on Human Care and 
Use of Laboratory Animals). The animals were anesthetized 
with 10 mg/kg ketamine intra-muscular, and 5 ml of sterile 
HIRMAb-PIL containing 70 µg of plasmid DNA was injected 
into the monkey via the saphenous vein with a 18-g catheter. 
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 
antibody. The injection dose of PIL encapsulated plasmid DNA 
was 12 µg/kg. The eyes were enucleated immediately after 
euthanasia. In addition, the brain, liver, spleen, lung, heart, 
kidney, triceps skeletal muscle and omental fat were removed. 
The conjunctiva, orbital connective and muscular tissues, and 
vitreous body were removed from the eye. One eye was used 
for measurement of luciferase activity and the other eye was 
frozen in powdered dry ice for 30 min, embedded in OCT and re-frozen for cryostat sectioning. Eyes were also removed from a control rhesus monkey (Sierra Biomedical/Charles River; Sparks, NV), not injected with gene, and these eyes were frozen in OCT embedding medium immediately after euthanasia, and processed in parallel with the eyes from the gene injected monkey.

The experimental design involved the injection of either 
1 or 2 plasmids in the same animal, so as to minimize the number of terminal primate experiments. Monkey 1 was injected on day 0 with both clone 756 (the SV40 driven β-galactosidase plasmid), and clone 790 (the SV40 driven luciferase plasmid), and sacrificed at 2 days after injection. For the monkey 1 experiment, the clone 756 or clone 790 plasmid DNA was individually encapsulated in separate preparations of HIRMAb-targeted PILs and co-injected into the primate, similar to the experimental design used previously [14]. Monkey 2 was injected with clone 790 on day 0, and injected with clone 933, the pLacF (the rhodopsin promoter driven β-galactosidase plasmid), on day 5, and sacrificed on day 7. Monkey 3 was injected with clone 790 on day 0 and sacrificed on day 14. Monkey 4 was the control, uninjected primate.

β-Galactosidase histochemistry: Frozen sections of 20 µm thickness were cut on a Mikron cryostat and fixed with 0.2% glutaraldehyde in 0.1 M NaH₂PO₄ for 1 h. The sections were washed with 0.1 M NaH₂PO₄ three times, and incubated overnight at 37 °C in X-gal staining solution (20 mM potassium-ferrocyanide, 20 mM potassium-ferricyanide, 2 mM...
MgCl₂, 0.02% IGEPAL CA-630, 0.01% Na deoxycholate, and 1 mg/ml X-gal in 0.1 M NaH₂PO₄ at pH 7.3. Prior to coverslipping, the sections were scanned with a UMAX PowerLookIII scanner with transparency adapter, and the image was cropped in Adobe Photoshop 5.5 on a G4 Power Macintosh computer. Control or un-injected rhesus monkey eye was stained in parallel with the eye obtained from the gene-injected animal. Specimens were examined with and without hematoxylin counter-staining.

Confocal Imaging: Frozen sections (20 µm) of either gene-injected and control monkey eyes were fixed for 5 min in 100% acetone at room temperature (RT). Following air drying, all sections were washed in 0.01 M PBS buffer and non-specific binding was blocked using 10% goat serum in 0.01 M PBS with 0.1% Triton X-100, pH 7.4 for 60 min at RT. Primary antibodies were the 83-14 mouse anti-human insulin receptor, rabbit anti E. coli β-galactosidase polyclonal antibody (Biodesign Int., Saco, ME), mouse IgG2a isotype control, or rabbit IgG, 5 µg/ml, in 3% bovine serum albumin, 0.01 M PBS with 0.1% Triton X-100, pH 7.4; all primary antibodies were used at 5 µg/ml. Incubation time was 48 h at 4 °C in a humidified chamber. Secondary antibodies used were 594 Goat anti-mouse IgG and 488 goat anti-rabbit IgG (Molecular Probes; Eugene, OR), 5 µg/ml, in 1% goat serum, 0.01 M PBS, 0.1% Triton X-100, pH 7.4. Incubation time was 1 h at RT. Confocal imaging was performed with a Zeiss LSM 5 PASCAL confocal microscope with dual argon and helium/neon lasers equipped with Zeiss LSM software for image reconstruction (Zeiss, Jena, Germany). All sections were scanned in multitrack mode to avoid overlap of the fluorescein (excitation at 488 nm) and rhodamine (excitation at 543 nm) channels employing the Plan-Neofluar 40x/0.75 objective. Pinhole size was 126 µm in both channels, and detector gain, amplifier gain and amplifier offset was identical for the gene-injected and control retina. Two scanning lines were integrated for a 512x512 image matrix. By integrating 4 serial tomographic images stack sizes of 230.3x230.3x4.8 µm dimension were created. 2D images were visualized by integrating 4 serial tomographs by maximum intensity projection.

Luciferase measurements: Primate retina was homogenized in 4 volumes of lysis buffer for measurement of luciferase activity, as described previously [19]. The data are reported as pg luciferase activity per mg cell protein. Based on the standard curve, 1 pg of luciferase was equivalent to 14,312±2,679 relative light units (RLU), which is the mean±S.E. of 5 assays.

RESULTS

Histochemistry of the retina obtained from a control, un-injected Rhesus monkey shows no measurable β-galactosidase histochemical product (Figure 1A). In contrast, there is global β-galactosidase histochemical product in the primate retina and eye obtained 48 h after the single intravenous injection of the pSV-β-galactosidase plasmid encapsulated in the HIRMAb-PIL (Figure 1B). Exogenous gene expression is detected in multiple structures of the eye including the entire retina, the epithelium of the cornea, the ciliary body, and the iris. Higher magnification of the retina shows no evidence of histochemical product in the eye obtained from the un-injected monkey (Figure 1C), although there is gene expression in most layers of the retina in the gene-injected Rhesus monkey (Figure 1D). There is abundant histochemical product in the cell bodies of the ONL as well as the inner segments (IS) and some measurable histochemical product in the outer segments (OS) of the photoreceptor cells (Figure 1E). Other layers of the retina that are positive for β-galactosidase gene expression include the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL) and the ganglion cell layer (GCL); see Figure 1D.E. There is no structural damage to the retina caused by the PIL administration as shown by the counter-staining of the primate retina (Figure 1F).

The luciferase gene was also expressed in the retina, and the retinal luciferase activity at 48 h was 9.6±0.4 pg luciferase/mg protein (mean±S.E., n=3 replicates). This is equivalent to 1.4x10⁵ relative light units (RLU)/mg protein (Methods). No luciferase enzyme activity was detected in the retina of the control, un injected monkey. The retina luciferase activity was also measured in primates at 7 and 14 days after injection, and the retinal luciferase activity decayed exponentially (Figure 2). The half-time of luciferase gene expression decay was 2.0±0.1 days and the extrapolated Y-intercept [A(0)], which

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Figure 2. Luciferase gene expression in the primate eye. Retinal luciferase activity in 3 rhesus monkeys sacrificed at 2, 7, or 14 days after intravenous administration of clone 790 in monkeys 1, 2, and 3, respectively (see Methods). The data were fit to a single-exponential equation to yield the slope/half-time (t₁/₂) and the intercept, [A(0)]. The horizontal line shows the A(0) of luciferase activity in control mouse brain, 0.22±0.08 pg/mg [20], which indicates the level of gene expression in the monkey retina is still above the therapeutic level for at least 2 weeks after injection. The luciferase expression plasmid was under the influence of the SV40 promoter in these studies. The diagonal line through the data points was drawn by eye. Error bars representing the standard deviation are shown over each closed circle.
gives the maximal retinal luciferase level, was 19.3±0.1 pg per mg protein (Figure 2). The horizontal line in Figure 2 is the luciferase A(0) in the mouse [20], which corresponds to the primate retina luciferase activity at 12 days after a single intravenous injection (Figure 2).

Confocal microscopy shows diffuse expression of the IR in the retina of the gene-injected primate with high levels in the IS, OPL, IPL, and intermediate levels of expression in the INL and ONL cell bodies (Figure 3A). The same pattern of retinal expression of the IR is seen in the control monkey (Figure 3D). There is diffuse immunoreactive β-galactosidase in the gene-injected monkey with high levels in the IS, OPL, and IPL (Figure 3B). There is no immunoreactive β-galactosidase in the un-injected control monkey, as the faint autofluorescence seen over the RPE, OPL, and rod outer segments (Figure 3E, left lower corner) was also detected with control rabbit IgG (Figure 3H). The IR/β-galactosidase overlap images for the gene-injected monkey and un-injected control monkey are shown in Figure 3C,F, respectively. There is a co-localization of β-galactosidase and IR in the gene injected monkey with minimal overlap signal in the un-injected monkey (Figure 3F).

The organ specificity of β-galactosidase gene expression was examined in the primate at 2 days following the intravenous injection of the pLacF plasmid driven by the bovine opsin promoter. Although there was abundant gene expression in multiple structures of the eye (Figure 4A), there was no gene expression in monkey brain, heart, lung, fat, spleen, or liver (Figure 4C-H). The negative histochemical reaction in these multiple peripheral tissues was not assay related, because the histochemistry was done in parallel with the primate eye (Figure 4A,I) as well as the primate kidney (Figure 4B,J). Control kidney from un-injected animals contains β-galactosidase that is active at neutral pH and is histochemically active in the β-galactosidase assay [7]. Therefore, the histochemistry of kidney tissues serves as a positive control for the β-galactosidase assay. The pattern of cellular gene expression in the layers of the primate retina following administration of the opsin promoter plasmid (Figure 4I) is comparable to that found with the SV40 promoter plasmid as shown in Figure 1E. β-galactosidase gene expression under the influence of the opsin promoter was observed in multiple layers of the retina including the ONL, and the IS and OS of the photoreceptor cells (Figure 4I).

**DISCUSSION**

The results of these studies support the following conclusions. First, exogenous plasmid-based genes may be targeted to nearly all structures of the primate eye using the PIL gene targeting system and a HIRMAb as the targeting ligand (Figure 1). Second, the IR is expressed in the cell bodies of the ONL (Figure 3A), which enables global expression of exogenous genes in the photoreceptor cells and particularly IS (Figure 1E and Figure 3B). Third, β-galactosidase gene expression in the rhesus monkey is restricted to the eye when the trans-gene is under the influence of a ocular-specific promoter such as the opsin.
promoter (Figure 4). Fourth, the expression of a reporter gene in the monkey eye declines following a single intravenous injection of plasmid and decays exponentially with a $t_{1/2}$ of 2.0 ± 0.1 days (Figure 2).

The nearly global expression of the exogenous gene delivered to the primate eye via the HIRMAb targeted PIL is shown by the β-galactosidase histochemistry (Figure 1B). There is gene expression in the retina, the cornea, the ciliary body, and the iris, and this global expression in the eye is observed with either the SV40 promoter (Figure 1B) or the bovine opsin promoter (Figure 4A). The sites of β-galactosidase gene expression parallel the sites of expression of the insulin receptor. The IR is expressed in the human eye in the epithelium and endothelium of the cornea, the lens capsular epithelium, and multiple cells within the retina including the inner segments, the ONL, the RPE, GCL, and Müller cells [11,12]. In addition to the widespread expression within the eye of the IR, this receptor is also useful for gene targeting to the nucleus. The IR serves to deliver ligand to the nucleus; prior work has shown that the IR delivers to the nucleus the majority of plasmid DNA taken up by the cell [19,21]. The mechanism of gene delivery to the eye is receptor-mediated, and not a nonspecific breakdown of the BRB caused by the PIL injection. Prior work has shown that when the β-galactosidase expression plasmid is encapsulated in a PIL targeted with an isotype control antibody that does not recognize any receptor, then there is no β-galactosidase gene expression observed either in the eye [6], or in any other organ [7,8]. There is normal cellular architecture in the retina following PIL administration (Figure 1F).

The present studies target genes to the primate eye with the HIRMAb and demonstrate gene expression in the primate photoreceptor cells (Figures 1, 3 and 4). In contrast, prior work in mice targeted genes to the retina with the TfRMAb, and no expression of the β-galactosidase gene in the photoreceptor cells of the mouse retina was observed [6]. This observation correlates with the known tissue-specific expression of the TfR within the retina. The TfR is produced in the RPE, IS, OPL, INL, and GCL, but is minimally expressed in the cell bodies of the ONL [9,10]. The low expression of TfR in the ONL is consistent with the very low stores of either iron or ferritin in the ONL [10]. The TfR is expressed on the IS of the photoreceptor cells [6], but PIL entry into the photoreceptor cell at the IS apparently cannot support gene expression [6]. There may be minimal retrograde transport of the PIL from the IS to the cell body of the photoreceptor cell in the ONL. While the TfR is minimally expressed in the cell bodies of the ONL [10], the insulin receptor is expressed in this region of the retina in both humans and rats [11,12]. The present studies demonstrate that the ONL of the primate retina is also a site of abundant insulin receptor expression as shown by confocal microscopy (Figure 3A,D). In parallel with the expression of insulin receptor at the ONL, the β-galactosidase gene is expressed in the photoreceptor cells of the monkey (Figure 1E). The β-galactosidase gene expression is more prominent in the inner segments relative to the outer segments, although β-galactosidase activity in the outer segments is visible by histochemistry (Figure 1E and Figure 4I).

The organ specificity of gene expression is a function of two variables: (a) the organ specificity of the targeting MAb attached to the PIL, and (b) the organ specificity of the pro-
moter incorporated in the expression plasmid. In prior work in the rhesus monkey, the pSV-β-galactosidase expression plasmid was under the influence of the widely expressed SV40 promoter. Therefore, gene expression was observed in multiple organs of the primate including brain, liver, and spleen [14]. However, the present studies show that the expression of the β-galactosidase gene is restricted to the eye when the β-galactosidase expression plasmid is under the influence of the bovine opsin promoter (Figure 4). The pLacF β-galactosidase expression plasmid is driven by the 2 kb of the 5'-flanking sequence (FS) of the bovine opsin gene [16]. Under the restriction of the opsin promoter, there is no expression in brain, liver, spleen or other organs following the intravenous administration of HIRMAb-targeted PILs in the rhesus monkey (Figure 4). This observation in the primate of organ-specific gene expression with organ-specific promoters parallels prior work in the mouse and rat [7,8]. Genes driven by the SV40 promoter and encapsulated in TIRMAb-targeted PILs are expressed in multiple TIR-rich peripheral organs including brain, liver, and spleen [7]. However, a different pattern of gene expression is observed if the exogenous gene is driven by a brain-specific promoter such as the 2 kb of the 5'-FS of the glial fibrillary acidic protein (GFAP) gene. If the SV40 promoter is replaced with the GFAP promoter, then gene expression in the brain and eye is preserved [6,7], whereas trans-gene expression in peripheral tissues such as liver or spleen is eliminated [7]. The finding of eye-specific gene expression in the monkey with the pLacF plasmid (Figure 4) parallels prior work with transgenic mice produced with the pLacF gene. Gene expression is confined to the eye in either the germ cell transgenic mouse [16], or the adult rhesus monkey made acutely transgenic with the PIL gene transfer technology (Figure 4).

The bovine opsin promoter restricts transgene expression to the eye, but does not restrict gene expression to the photoreceptor cells in the monkey (Figure 4A). A similar finding was made in transgenic mice produced with the pLacF construct, as these transgenic mice expressed the β-galactosidase gene in the photoreceptor cells, as well as the iris and ciliary body, and the brain [16]. One possible explanation for the diffuse expression of the reporter gene in the eye is selective expression in the photoreceptor cells followed by secretion and diffusion to other parts of the eye. However, this explanation appears unlikely since prior work has shown selective localization of the β-galactosidase enzyme in retinal structures without secretion and diffusion to other parts of the eye [6]. A more likely explanation for the broad spectrum of opsin promoter driven gene expression in the eye is that the other ocular structures are embryologically related to the neural retina. Transfection of iris or ciliary body with the photoreceptor cell specific Crx homeobox gene results in the synthesis of rhodopsin in these extra-retinal ocular structures [22]. The restriction of gene expression to the photoreceptor cells may require regulatory gene elements in addition to the 5'-FS of the gene. In the case of brain GFAP gene expression, the 5'-FS confers brain specificity, but does not restrict gene expression within the brain to astrocytes. Astrocyte specific gene expression requires the coordinate interaction of gene elements in both the 5'-FS and the 3'-FS of the GFAP gene [23,24].

The luciferase gene expression decays exponentially with a half-time of 2.0±0.1 days, and the peak luciferase expression, A(0) is 19.3±0.3 pg/mg (Figure 2). The peak luciferase gene expression is 50-fold higher than the A(0) in rodent brain [14,20], and the rodent A(0) is shown by the horizontal line in Figure 2. The higher peak level of gene expression in the primate, relative to the rodent, is attributed to the much higher activity of the HIRMAb as a targeting ligand, as compared to the TIRMAb [21]. The HIRMAb is used to target PILs to primate brain [14], whereas the TIRMAb is used to target PILs to rodent brain [6-8]. The level of gene expression achieved with the TIRMAb is sufficient to cause the desired pharmacological effect. The intravenous administration of TIRMAb-targeted PILs produces a 100% increase in survival time in mice with experimental brain cancer treated with EGFR antisense gene therapy [20], or a 100% normalization of striatal enzyme activity in rats with experimental Parkinsonism treated with tyrosine hydroxylase gene therapy [25]. Therefore, the mouse A(0) shown in Figure 2 represents levels of gene expression that yield therapeutic effects. Owing to the very high initial level of gene expression in the primate with the HIRMAb, the level of retinal gene expression is still in the therapeutic range for more than 2 weeks after a single intravenous injection (Figure 2). Therefore, primates may require repeat administration of gene therapy at intervals of 3-4 weeks.

In summary, these studies show it is possible to achieve global expression of an exogenous gene in photoreceptor cells of the primate retina following a non-invasive, intravenous administration of a non-viral form of the gene. The plasmid replicates episomally and gene expression is reversible (Figure 2). Southern blotting shows no integration of the plasmid DNA into host chromosomal DNA following PIL gene delivery [8]. Similarly, plasmid DNA delivered to organs in vivo with the hydrodynamics injection method is not integrated in the host chromosome [26]. Owing to the transient nature of plasmid-based gene expression, it is necessary to administer the PIL formulation at regular intervals to sustain a therapeutic effect, and this has been done for the treatment of mice with brain cancer [20]. The chronic weekly intravenous administration of PIL encapsulated genes causes no change in organ histology, serum chemistry, or body weights, and causes no inflammation in the central nervous system [27]. Repeat intravenous administration of the PIL formulation is possible, because the only antigenic component of the liposome is the targeting ligand. The immunogenicity of the targeting MAb can be reduced or eliminated with genetic engineering and “humanization” of the original murine MAb. The murine 83-14 HIRMAb has been genetically engineered to produce the chimeric form of this antibody, and the chimeric HIRMAb has an identical affinity for the HIR as the original murine antibody [28]. The chimeric HIRMAb rapidly crosses the primate BBB in vivo [28], and the present studies provide evidence that the HIRMAb also crosses the primate BRB and distributes to the retina and to multiple ocular structures.
ACKNOWLEDGEMENTS

Dafang Wu, Hwa Jeong Lee, Chunni Zhu, Toyofumi Suzuki, and Yufeng Zhang of the UCLA Department of Medicine provided assistance in the primate experiments. This work was supported by grants from the University of California, Davis/Medical Investigation of Neurodevelopmental Disorders Institute Research Program, and the Neurotoxin Exposure Treatment Research Program of the U. S. Department of Defense. Felix Schlachetzki was supported by a grant from the Ernst Schering Research Foundation (Berlin, Germany). This work was presented in part at the Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO-2003), Fort Lauderdale, FL, May, 2003.

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