Fetal Gene Transfer Using Lentiviral Vectors: \textit{In Vivo} Detection of Gene Expression by microPET and Optical Imaging in Fetal and Infant Monkeys

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ABSTRACT

Fetal intraperitoneal administration of human immunodeficiency virus (HIV)-1-derived lentiviral vectors (10^7 infectious particles/fetus) has consistently shown high levels of transduction and gene expression in the omentum, peritoneum, and diaphragm when assessed by polymerase chain reaction (PCR) and whole tissue fluorescence. \textit{In vivo} imaging techniques were explored with early-gestation long-tailed macaques that were administered the vesicular stomatitis virus-glycoprotein (VSV-G)-pseudotyped HIV-1-derived lentiviral vector expressing a mutant herpes simplex virus type 1 thymidine kinase (HSV-1-sr39tk) and firefly luciferase under the control of the cytomegalovirus (CMV) promoter. Fetuses were monitored sonographically and twice during gestation 9-[4-[18F]Fluoro-3-(hydroxymethyl)butyl]guanine (18F-FHBG) was injected into the fetal circulation under ultrasound guidance in preparation for microPET imaging. All newborns were delivered at term by cesarean section and raised in the nursery for postnatal studies. At 2 months postnatal age, animals were imaged and biodistribution was assessed. Optical imaging for firefly luciferase expression was also performed every 2 months postnatal age. Under all imaging conditions gene expression was observed in the abdominal region, and closely paralleled findings from prior studies based on whole tissue fluorescence. These investigations have shown that HSV-1-sr39tk and firefly luciferase can be used to safely detect transgene expression at multiple time points in fetal and infant monkeys \textit{in vivo} and without evidence of adverse effects.

OVERVIEW SUMMARY

Significant advancements have been made in the use of noninvasive imaging techniques to monitor gene expression using contrast-generating reporter genes. The goal of this study was to explore the use of microPET and optical imaging for monitoring gene expression in fetal and infant monkeys longitudinally and over time. The HIV-1-derived lentiviral vector expressing HSV-1-sr39tk and firefly luciferase under the control of the CMV promoter was administered in the late first trimester, and microPET imaging was performed twice during gestation. Beginning at 2 months postnatal age, then every 2 months thereafter, animals were imaged and biodistribution was assessed with microPET and/or optical imaging. Under all imaging conditions gene expression was found to persist in most animals, and without evidence of adverse effects.

INTRODUCTION

Significant advances have been made in the use of noninvasive imaging techniques to monitor transgene expression longitudinally \textit{in vivo} and over time. These techniques include nuclear imaging such as positron emission tomography (PET) that use radioactively tagged tracers (Phelps, 2000a; Cherry and Gambhir, 2001; Cherry, 2004) and optical techniques based on bioluminescence or fluorescence (Contag and Bachmann, 2002; Bremer \textit{et al.}, 2003). PET imaging has been used to assess the distribution of tracers labeled with radioisotopes \textit{in vivo} for a
wide variety of applications including the diagnosis of cancer and cardiovascular diseases, and for neurologic studies (Phelps, 2000b). The simultaneous emission of two 511-keV γ-rays from the annihilation of positrons in biologically active molecules labeled with radioisotopes allows spatial and functional imaging of biochemical reactions in vivo (Cherry and Gambhir, 2001).

Reporter genes for PET imaging include the mutant herpes simplex virus-thymidine kinase gene (HSV-1k); radiolabeled substrate for the enzyme is trapped only in cells that express the reporter gene (Gambhir et al., 2000b). Gambhir and colleagues have developed methods to image expression of the mutant HSV-1-sr39k gene delivered by viral vectors into rodents (Gambhir et al., 1999, 2000a; Yu et al., 2000). The ability to visualize, localize, and accurately quantify radiotracer accumulation that is reflective of a level of transgene expression is contingent on several factors, including the resolution and sensitivity of the imaging measurement, and for PET imaging, the pharmacokinetics of the radiotracer, in particular the kinetics of target accumulation versus excretion. For bioluminescence approaches, the reporter gene produces an enzyme that can catalyze a bioluminescent reaction in the presence of the necessary substrate, leading to the emission of light. A common reporter gene used is firefly luciferase and its substrate, luciferin.

The gene transfer efficiency of human immunodeficiency virus (HIV)-1-derived lentiviral vectors pseudotyped with vesicular stomatitis virus-glycoprotein (VSV-G) and driven by the myeloproliferative sarcoma virus (MND) or cytomegalovirus (CMV) promoter and expressing enhanced green fluorescent protein (EGFP) has been previously investigated in fetal and infant monkeys using systemic (Tarantal et al., 2001a; Jimenez et al., 2005) and organ-targeting approaches (Tarantal et al., 2001b, 2005). In one series of studies (Jimenez et al., 2005), early-gestation fetuses were administered lentiviral vector supernatant under ultrasound guidance, using an intraperitoneal approach with a range of 1 × 10⁷ to 2 × 10⁸ infectious particles/fetus. Analysis of transgene biodistribution and expression by polymerase chain reaction (PCR) and direct tissue fluorescence was consistently observed in the omentum, diaphragm, and peritoneum, with tissue fluorescence shown up to 3 years postnatal age (Fig. 1). On the basis of these findings our objective was to determine whether we could monitor gene expression in vivo using noninvasive imaging methods. In this study, we used an HIV-1-derived lentiviral vector with a dual reporter including HSV-1k and firefly luciferase (Ray et al., 2003; Min and Gambhir, 2004) that allowed the monitoring of transgene expression in animals after fetal gene delivery in vivo, using both optical imaging and microPET technologies.

The goals of this study were 2-fold. We first determined the safety and efficiency of microPET imaging in fetal macaques, and after direct fetal injection of 9-[4-[18F]fluoro-3-(hydroxymethyl)butyl]guanine ([18F]FHBG) under ultrasound guidance. Next, we explored the use of a combined microPET and optical imaging approach for postnatal analyses to correlate findings using the two imaging modalities, and to assess the feasibility of the use of these two techniques in young monkeys. These investigations have clearly shown that the HIV-1-derived lentiviral vector with a dual reporter driven by the CMV promoter can be used efficiently to monitor gene expression longitudinally and over time without adverse effects, and that in vivo imaging findings paralleled those that have previously been observed at the tissue level when using EGFP as a reporter gene.

**MATERIALS AND METHODS**

**Animals**

All animal procedures conformed to the requirements of the Animal Welfare Act and protocols were approved before implementation by the Institutional Animal Care and Use Committee (IACUC) at the University of California at Davis. Normally cycling, adult female long-tailed macaques (Macaca fascicularis) (n = 7; 3.5–5.4 kg) with a history of prior pregnancy were bred and identified as pregnant, using established methods (Tarantal, 2005). Pregnancy in long-tailed macaques is similar to rhesus macaques and is divided into trimesters by 55-day increments, with 0–55 days of gestation representing the first trimester, 56–110 days of gestation representing the second trimester, and 111–165 days of gestation representing the third trimester (term, 165 ± 10 days) (Tarantal and Gargosky, 1995). Rhesus and long-tailed macaques have long been used side-by-side experimentally for developmental and reproductive studies (Tarantal and Hendrickx, 1988a,b; Tarantal, 1990; Tarantal and Gargosky, 1995). This species was chosen for these studies because of body size and the bore size of the microPET scanner which permitted a comfortable fit during pregnancy.

**Lentiviral vector**

The HIV-1-derived lentiviral vector used for these studies (CS-CMVsr39k-I-Fluc) was kindly provided by S. Gambhir (De et al., 2003). The internal ribosomal entry site (IRES)-based bicistronic lentiviral vector includes HSV-1-sr39k in the first cistron and firefly luciferase (Fluc) in the second cistron, separated by a translational cis-acting element, the encephalomyocarditis virus (EMCV) IRES sequence, which allows translation of two open reading frames from a single mRNA.

**Vector administration and fetal monitoring**

All fetuses were sonographically assessed to confirm normal growth and development prior to gene transfer (Tarantal, 2005). The dams were administered ketamine hydrochloride (10 mg/kg, intramuscular) for these and subsequent ultrasound examinations. On the day of gene transfer, the dams (n = 5) were administered ketamine (10 mg/kg) or Telazol (5–8 mg/kg, intramuscular), and were aseptically prepared for transabdominal ultrasound-guided fetal gene transfer. A total volume of ~300 μl of the lentiviral vector supernatant was injected intraperitoneally using established techniques (Tarantal, 1990; Jimenez et al., 2005). All fetuses were monitored sonographically during gestation with measurements of the fetal head (biparietal and occipitofrontal diameters, area, and circumference), abdomen (area and circumference), and limbs (humerus and femur lengths), in addition to gross anatomical evaluations (axial and appendicular skeleton, viscera, membranes, placenta, and amniotic fluid) approximately every 10 days until term cesarean section delivery, as previously described (Tarantal and Hendrickx, 1988b; Tarantal, 2005). All measures were compared to normative growth curves for long-tailed macaque fetuses (Tarantal, 2005). Concurrent controls
Term delivery and postnatal sample collection

Newborns were delivered by cesarean section at term (160 ± 2 days of gestation). Cord blood samples (~12–15 ml) were collected at birth (CBCs, clinical chemistry panels, serum, plasma, and peripheral blood mononuclear cells [PBMCs]) using established protocols, and then simian Appgar scores and morphometrics (birth weight; crown–rump length; head, chest, and arm circumferences; humerus, femur, hand, and foot lengths; skinfolds) were assessed using standard techniques (Tarantal and Hendrickx, 1989). Newborns were placed in incubators postdelivery and nursery-reared up through 3 months postnatal age, and then moved into juvenile housing. Infant health, food intake, and body weights were recorded daily in the nursery and then on a routine basis according to established protocols. Blood samples (~3–6 ml, depending on age) were collected every 2 months from a peripheral vessel to monitor CBCs and clinical chemistry parameters and to preserve serum, plasma, and PBMCs.

microPET and optical imaging

The microPET P4 imaging system (CTI Molecular Imaging; Siemens Medical Solutions USA) used for these studies has a 22-cm bore, 20-cm transaxial field of view, and 8-cm axial field of view. The sensitivity of the unit is 2.25% at the center of the field of view with an energy window of 250–750 keV and a timing window of 10 nsec (default values). With maximum a posteriori (MAP) reconstruction incorporating an accurate system model (standard reconstruction algorithm used), image resolution is ~1.8 mm isotropically (6-pL volumetric resolution) (Tai et al., 2005). In these investigations we focused on identifying elevated accumulation of [18F]FHBG that would be indicative of expression of the HSV-1tk reporter gene. [18F]FHBG was synthesized at the LA Tech Center (Culver City, CA) or in the Center for Molecular Imaging (University of California, Davis), as previously described (Yaghoubi et al., 2001).

At 100 days of gestation (second trimester), animals (gene transfer and controls) were sedated with ketamine and then ~300–600 μCi of [18F]FHBG (0.4–0.6 ml) was injected directly into the fetal circulation under ultrasound guidance, using established techniques (Tarantal, 1990). The anatomical position of the fetus in relation to the dam as detected by ultrasound was used to determine the microPET scanning positions, and each dam was positioned on the scanning bed with three to five bed positions assessed (15 min per bed) 30 min after fetal injection. Fetal injection and imaging were repeated at 130 days of gestation (third trimester), using ~400 μCi per fetus (0.7 ml) and similar techniques. Images were reconstructed according to a MAP reconstruction algorithm. For the first group of animals (n = 3), imaging was performed with the animals maintained on isoflurane. After these initial scanning procedures, future scans were performed under ketamine for the imaging session done 15 min after [18F]FHBG injection and under Telazol for the imaging session performed 2–3 hr after [18F]FHBG injection.

At ~1–2 months postnatal age, infants were sedated with either ketamine or Telazol and, after an intravenous injection of 650 μCi (~3 ml) of [18F]FHBG, biodistribution was assessed. In vivo optical imaging for firefly luciferase expression was also performed beginning at 1–2 months of age, immediately after an intravenous injection of 1 ml of β-luciferin (100 mg/kg), using a Xenogen IVIS imaging system with Living Image software analysis (Xenogen, Alameda, CA). Typically, the optical imaging preceded microPET imaging on the days it was performed, and provided identification of areas of transgene expression that aided in choice of the microPET scanning bed positions. Bioluminescence imaging was repeated every 2–3 months whereas microPET scanning was performed every 6 months. For optical imaging, each animal was placed in dorsal recumbency for each imaging session in a light-tight chamber, and whole body images were obtained with quantification. Bioluminescent and photographic images were superimposed, using Living Image 2.50 software (Xenogen). Regions of interest (ROI) were defined by selecting areas showing bioluminescence. Total photons per second per square centimeter detected in ROI were recorded and compared between different animals and time points. Both gene transfer and control animals were imaged on the same day.

RESULTS

All newborns (n = 7) were delivered at term as scheduled, with no adverse effects detected. No prenatal or postnatal indices were found to differ when compared with concurrent or historical controls. CBCs, chemistry panels, and growth were all within normal limits (data not shown). Daily activity patterns were also within the anticipated range for all age groups. No abnormalities have been observed, to date (oldest animals are currently ~2 years postnatal age).

microPET imaging

In our prior studies, fetal intraperitoneal administration of HIV-1-derived lentiviral vectors expressing EGFP under the control of the CMV promoter resulted in expression of the transgene primarily in abdominal areas including the omentum, diaphragm, and peritoneum when assessed by whole tissue fluorescence (see Fig. 1), which was confirmed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) (Jimenez et al., 2005). In the current study, a bicistronic lentiviral vector expressing HSV-1-sr39tk and firefly luciferase under the control of the CMV promoter was administered to early-gestation fetal monkeys to monitor transgene expression in vivo. Fetal monkeys were imaged at 100 days (second trimester) and 130 days (third trimester) of gestation, 15 min and 2–3 hr after fetal injection of [18F]FHBG. Imaging indicated that the HSV-1-sr39tk gene was expressed exclusively in the abdominal region (Fig. 2). There was no evidence of adverse effects as a result of direct fetal injection of [18F]FHBG in any of the animals assessed. Postnatal studies were performed at selected time points and to correlate with specific optical imaging sessions (see below). microPET imaging was performed 15 min and 2–3 hr after intravenous injection of [18F]FHBG, as described above. Similar patterns of gene expression were observed in abdominal locations (see Fig. 3).

Postnatal optical imaging

The initial group of animals included three monkeys (two gene transfer, animals 1 and 2; one control, animal C1). Lucif-
erase expression was monitored postnatally by injecting animals with D-luciferin and detecting bioluminescence with the Xenogen IVIS imaging system. Similar to findings with microPET and prior studies assessing whole tissue fluorescence, transgene expression was observed predominantly in the abdominal area (Fig. 4). Initially, infant 1 showed a greater level of bioluminescence ($7.4 \times 10^8$ photons/sec; Fig. 4E) compared with infant 2 ($1.0 \times 10^8$ photons/sec; Fig. 4I) at 2 months postnatal age. However, the level of bioluminescence in infant 1 dramatically declined to $2.6 \times 10^6$ photons/sec (Fig. 4F) after 2 months, whereas infant 2 showed stable gene expression ($1.2 \times 10^9$ photons/sec; Fig. 4J). The levels of bioluminescence in animal 2 further declined to $4.1 \times 10^7$ photons/sec at 1 year postnatal age (15 months after gene transfer) (Fig. 4K) and to $7.3 \times 10^7$ photons/sec at 1.5 years postnatal age (21 months after gene transfer) (Fig. 4L). Interestingly, the level of luciferase expression in infant 1 remained at $3.0 \times 10^6$ photons/sec at 1 year postnatal age (15 months after gene transfer) (Fig. 4K) and to $7.3 \times 10^7$ photons/sec at 1.5 years postnatal age (21 months after gene transfer) (Fig. 4L).}

The second group of four animals (three gene-transferred, animals 3, 4, and 5; one control, animal C2) showed a similar outcome, with some variation in the gene expression profiles. This group of animals was monitored up to 9 months postnatal age (current age; 12 months post-gene transfer). Infant 3 ($8.1 \times 10^9$ photons/sec; Fig. 5E) and infant 5 ($6.1 \times 10^8$ photons/sec; data not shown) showed similar levels of luciferase expression at 1 month postnatal age, whereas a lower level of transgene expression was observed in infant 4 ($2.6 \times 10^8$ photons/sec; Fig. 5I) at the same time point. Infant 3 maintained approximately the same levels of luciferase expression throughout the study period (Fig. 5F–H). Interestingly, higher levels of luciferase expression were observed in infant 5 at 3 months ($1.9 \times 10^7$ photons/sec; 6 months, $1.1 \times 10^9$ photons/sec; and 9 months, $8.2 \times 10^8$ photons/sec postnatal age (12 months after gene transfer) (data not shown). These find-
ings may be due to some positional differences when comparing each of the imaging sessions. Animal 4 showed a decline in the levels of bioluminescence, similar to infant 2 (see Fig. 4). Overall, many of the animals included in this study showed high levels of gene expression throughout the duration of the imaging sessions, and with no evidence of adverse effects. Similar to our prior findings, the location of the fetal intraperitoneal injection was evident by the region of the bioluminescence profile.

Images of transgene expression acquired by bioluminescence and microPET combined indicated similar findings, with a primary focus of gene expression noted in the right upper quadrant in the animal shown in Fig. 3, although some of the areas of expression of luciferase noted with optical imaging (right ovary) were not observed with microPET at the later imaging sessions.

**DISCUSSION**

These studies describe for the first time successful methods for in vivo imaging of fetal and infant monkeys with microPET and bioluminescence. Findings have also shown that direct injection of [18F]FHBG and luciferin into the fetal and infant circulations does not result in adverse effects and provides good imaging outcomes. Whereas techniques for monitoring luciferase expression have been well developed, the techniques for microPET imaging will require further refinement in order to obtain correlative information at the level necessary. [18F]FHBG was not sufficiently cleared from abdominal organs before signals from transduced tissues could be clearly differentiated from background levels. Thus, we are currently exploring methods to ensure that the imaging results obtained clearly represent transgene expression.

In the current study, transgene expression was safely monitored in fetal monkeys, using [18F]FHBG as a substrate for HSV-1-sr39tk. Transgene expression was observed predominantly in the abdominal region and correlated with findings from our prior studies at the tissue level (Tarantal et al., 2001a,b; Jimenez et al., 2005). Similar to findings in rodents, we were able to achieve good volumetric spatial resolution. PET has also been used to investigate maternal–fetal exchange of drugs in nonhuman primates, combined with magnetic resonance imaging (MRI) (Benveniste et al., 2003, 2005), but these studies were accomplished by maternal injections of the substrate. In the current study, the injection of [18F]FHBG into the fetal circulation resulted in a unique image of the fetus with accumulation of the radiotracer in the maternal bladder (data not shown), indicating that [18F]FHBG can readily cross the placenta by retrograde transport and without maternal or fetal adverse effects (data not shown). These studies indicate the feasibility of using microPET to study transplacental (bidirectional) trafficking of cells, nutrients, potential toxic materials, metabolites, and drugs in a noninvasive manner.

Because of low intrinsic bioluminescence in mammalian tissues, a high signal-to-noise ratio can be achieved with in vivo optical imaging using bioluminescence reporters, most commonly luciferase cloned from the North American firefly (Photinus pyralis) and sea pansy (Renilla). The biochemical reaction of luciferin catalyzed by luciferase emits a broad spectrum of light (530–640 nm), and spectral content above 600 nm can penetrate through tissues with an approximate 10-fold loss of photon intensity for each centimeter (Sadikot and Blackwell, 2005). In a murine model, approximately 15–25% of light was shown to transmit through the skin, whereas only 1–2.5% of light transmitted from deep tissues such as the lung (Zhao et al., 2005). This characteristic absorption of bioluminescence by mammalian tissues may be problematic when imaging larger animals such as nonhuman primates. In this study, three of five gene-transferred monkeys showed good levels of luciferase expression, whereas detectable photons were not observed in two animals by 3 or 9 months postnatal age. Although variation in the levels of transgene expression between different animals has been seen in our studies with lentiviral vectors, none of the animals showed complete silencing of the transgene when analyzed by PCR (Tarantal et al., 2001a,b; 2005; Jimenez et al., 2005). The sensitivity of the CCD camera used for optical imaging and low levels of transgene expression in deep tissues could explain reduced detection of bioluminescence in two animals at the later time points. Thus, the visualization and quantification of bioluminescence may not always represent actual luciferase activity in vivo because of the attenuation of light through tissues, and particularly when the levels of expression are low. Despite these limitations, this study clearly indicates that bioluminescence imaging can be used efficiently and safely to monitor transgene expression in young nonhuman primates, and to compare the level of gene expression over time in vivo. Although the bioluminescent signal attenuation through animal tissues may be greater in nonhuman primates when compared with mice because of their size, the optical imaging method provides a relatively fast and efficient way to monitor transgene expression.

Semi-quantitative numbers were obtained from bioluminescence images by drawing ROI around the whole animal image obtained. These numbers were used to compare overall levels of gene expression over time and between different animals. It was also possible to draw ROI around regions with specific levels of gene expression. However, this method generated numbers that were highly variable, which may be due to the imaging position and overall growth of animals during the course of the study. Thus, semi-quantitative numbers obtained from whole animal images may provide more relevant information that could be used to compare the level of transgene expression over time and between animals in different study groups. Although silencing of the CMV promoter may explain the decreasing level of transgene expression observed during the initial period of this study, it is also possible that the increasing thickness of tissues that occurs with growth, and thus overlaying transduced cells, may interfere with the ability of bioluminescent signals to penetrate sufficiently. This emphasizes the importance of imaging animals in similar positions at each imaging session. It is important to note that the body wall of young monkeys is typically much thinner than that of humans (in the millimeter range), and thus the signal attenuation by overlaying tissues may not be substantial. However, loss of signal may be possible even with a slight increase in thickness of the overlaying tissue. Although the effects of lentiviral transduction and over-expression of transgenes under the control of a strong promoter, such as the CMV promoter, on the proliferation of cells in vivo has not been shown, it is possible that nontransduced cells may
FIG. 4. Postnatal optical imaging (group 1). Control (A–D) and gene-transferred animals (animals 1 and 2) (E–L) were imaged with the Xenogen IVIS imaging system immediately after injecting 1-luciferin (100 mg/kg, intravenous) at 2 months (A, E, and I), 6 months (B, F, and J), 12 months (C, G, and K), and 18 months (D, H, and L) postnatal age. No background was detected in the control animal at any time point (A–D). A high level of luciferase expression was initially detected in infant 1 (E) at 2 months of age, with a dramatic decline thereafter (F–H). Infant 2 showed stable gene expression at all time points (~21 months after gene transfer) (I–L).

FIG. 5. Postnatal optical imaging (group 2). Three additional gene-transferred animals (animals 3–5; only animals 3 and 4 are shown) and one control (A–D) were imaged at 1 month (A, E, and I), 3 months (B, F, and J), 6 months (C, G, and K), and 9 months (D, H, and L) postnatal age. No background signals were detected in the control animal at any time point assessed (A–D). Stable luciferase expression was observed in animal 3 (E–H) and detectable levels of gene expression were observed in animal 4 up to 6 months of age (I–K), with a dramatic decline at 9 months (L).
have a growth advantage relative to transduced cells. If a growth advantage did exist, one would also expect to see a diminished level of transgene expression over time in vivo with expansion of nontransduced tissue. These issues are important to consider when interpreting semiquantitative numbers obtained from bioluminescence images, such as those shown in these studies.

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REFERENCES


