AAV liver transduction efficiency measured by in vivo ^{18}F-FHBG-PET imaging in rodents and non human primates.

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Abstract

Recombinant adeno-asssociated virus 5 (rAAV5) represents a candidate vector with unique advantages for the treatment of hepatic disorders due to its narrow hepatic tropism. Non-invasive *in vivo* imaging of transgene expression provides a very important tool to quantify the transduction efficiency, duration and location of transgene expression. In this study, we used PET and PET-CT imaging to monitor the liver transduction efficacy in rodents and non-human primates that received rAAV5 vector encoding the herpes simplex virus thymidine kinase (TK). TK expression in the liver was also measured using immunohistochemistry. Notable differences in liver transduction efficiency are found dependent on the animal species and gender. Male rodents are better transduced than females, as previously described. Moreover, male non-human primates also displayed increased hepatic expression of the rAAV5-delivered transgene indicating that differences in rAAV mediated liver transduction can be anticipated in Man. Our results demonstrate high sensitivity and reproducibility of PET, using HSV-TK and ^{18}F-FHBG, to detect gene expression after rAAV vector administration into living animals, confirming the utility of this technology in the quantification of transgene expression, even at low expression levels. However, we also describe how an immune response against HSV-TK hampered analysis of long term expression in non-human primates.

Introduction

Recombinant adeno-associated viral (rAAV) vectors are currently the most frequently used viral vectors for clinical gene therapy (Grieger and Samulski, 2005; Daya and Berns, 2008). The lack of pathogenicity of rAAV vector results in persistent, long term expression, which combined with its relative non-immunogenicity have contributed to their increased popularity and use. Therapeutic genes carried by rAAV vectors have been efficiently delivered to various tissues leading to stable expression (Luebke *et al*., 2009; Stieger *et al*., 2009; Terzi *et al*., 2008; Markakis *et al*., 2010; Alexander *et al*., 2008). AAV2 is the best characterized serotype recombinant vector and has been used in over 40 clinical trials (Mueller and Flotte, 2008). However, the clinical efficacy of this vector is hampered by several limitations. Human populations are natural hosts for AAV2 and primary infection often generates anti-AAV2 neutralizing antibodies, which are prevalent in the general population (Boutin *et al*., 2010). For administration of rAAV2 vectors to the liver, the limited transduction efficiency is insufficient for some applications (Miao *et al*., 2000; Nakai *et al*., 2002). Moreover, rAAV2 has been used in a clinical trial for haemophilia B where a cytotoxic immune response against the AAV2 capsid was generated in some of the patients resulting in the elimination of rAAV2-transduced hepatocytes (Manno *et al*., 2006). Thus, the use of alternative AAV serotypes in humans might not only enhance the therapeutic efficacy, due to increased transduction of hepatocytes, but could also avoid the immune responses due to pre-existing immunity (Rivière *et al*., 2006; Peden *et al*., 2004). In this sense, AAV5, the most divergent of the AAVs (Gao *et al*., 2004) is a promising candidate since the prevalence of both IgG and neutralizing factors to AAV5 in the human population are the lowest compared to rAAV vectors with pseudotyped capsids 1, 2, 6, 8 and 9 (Boutin *et al*., 2010). This is particularly important since data indicate that even low levels of

neutralizing antibodies can completely abrogate transduction with high titers of vectors (Murphy *et al*., 2008). Furthermore, rAAV5 has a narrow tropism for the liver after intravenous delivery in mice (Pañeda *et al*., 2009). The liver tropism for AAV5 can be explained due to it's its receptor, platelet derived growth factor receptor (PDGFR) which is expressed at high levels on hepatocytes (Di Pasquale *et al*., 2003). However, as has been previously shown by different groups, significant differences in transduction efficiency and biodistribution can be observed depending on the animal species (Nathwani *et al.*, 2006; Davidoff *et al.*, 2005; Gao *et al*., 2006a). To develop safer and efficient gene delivery vectors, it is essential to perform thorough *in vivo* experiments, including the use of imaging technologies to identify rAAV patterns of transduction and to quantify transgene expression within different animal species, especially, non-human primates.

The current, most widely used modality to analyse viral vector efficiency and tropism *in vivo* is the construction of gene transfer vectors expressing the reporter gene luciferase, whose expression is monitored using live animal bioluminescence imaging (Pañeda *et al*., 2009). This technique has a high sensibility and specificity; however, these optical techniques are limited due to limited tissue penetration and detailed spatial resolution (Waerzeggers *et al*., 2009).

Nuclear medicine techniques, such as positron emission tomography (PET) imaging, enable evaluation of gene expression *in vivo* based on positron emitting radioisotopes, offering the possibility to monitor the exact location, magnitude and persistence of reporter gene expression. Among the many reporter genes suitable for PET imaging, the thymidine kinase gene from herpes simplex virus 1 (HSV1-TK) has been extensively exploited for imaging gene expression (Willmann *et al*., 2008). Imaging of HSV1-TK gene expression relies on the use of acycloguanosines, or uracil, derivatives labelled

with positron emitters. These molecular probes can traverse the plasma membrane by means of active transport, and are then trapped within the cell via phosphorylation by the viral TK (Yaghoubi and Gambhir, 2006). Cellular retention of radioactivity is, therefore, an indicator of gene expression of TK and can be detected by PET. Fluorine-18-labeled penciclovir analogue 9-(4-(18)F-Fluoro-3-[hydroxymethyl]butyl)guanine or ^{18}F-FHBG has been used as an effective substrate for HSV1-TK and evaluated as superior to other agents in many imaging applications and even used in humans (Gambhir *et al*., 1999; Peñuelas *et al*., 2002; Peñuelas *et al*., 2005a; Peñuelas *et al*., 2005b; Fontanellas *et al*., 2009). The development of micro-PET tomographs with a higher resolution has made this technology accessible for non-invasive and quantitative analysis of gene expression in long term animal studies (Del Guerra *et al*., 2002)). Integrated PET-computerized tomography (CT) machines combine in a single device a PET tomography and a CT. Both applications are performed simultaneously in the same individual in a single session. PET-CT can be more informative when identifying an anatomic-metabolic correlation to specific locations (Cherry, 2009).

In this study, we used PET imaging to monitor the expression of HSV1-TK in mice, rats and monkeys after intravenous injection of a rAAV5 encoding this TK under the control of a chimeric liver specific promoter (Kramer *et al*., 2003). In non-human primates we have combined the use of microPET with clinical PET/CT to determine the biodistribution of the radiotracer. TK expression in the liver was analysed by immunohistochemistry. Our data show that rAAV5-EalbAAT-TK delivered and mediated expression of TK in the liver. We also show that microPET is highly sensitive when analysing TK expression in rodents and non-human primates. Furthermore, there is a high correlation between TK expression analysis by PET and by

immunohistochemistry. The only limitation of using TK as a long term expression reporter gene is the induction of immune responses in non-human primates.

Materials and Methods

Animal Experiments

Male and female C57BL/6 mice (of 8 weeks of age and 24-26 g in weight) and Sprague Dawley rats (of 8 weeks of age and 160-170 g in weight) were obtained from Harlan Laboratories (Barcelona, Spain). Recombinant AAV were injected intravenously, via the tail vein, in 200 µl of saline solution for mice and 500 µl for rats.

Four young adult cynomologous monkeys (*Macaca fascicularis*) of similar age (3 years old), with body weights ranging from 2.5 to 4 kg, were obtained from R.C. Hartelist, (Tilburg, The Netherlands). Animals were kept in single cages and fed with food and water ad libitum, in standard facilities with controlled temperature and humidity, with a 12 h on-off light cycle. Animals were anesthetized by intramuscular injections of a mixture of 10 mg/kg ketamine (Imalgene) and 0.8 mg/kg midazolam (Dormicum). rAAV-EalbAAT-TK vector was administered via the saphenous vein, in a final volume of 10 ml, at two different doses 1×10^{12} vg/kg and 2.5×10^{12} vg/kg. Three weeks and two months after injection two liver biopsies were obtained by guided ultrasound imaging, under general anesthesia. After biopsy, monkeys received an intramuscular injection of Ketofen (5 mg/kg) and enrofloxacine antibiotic (10 mg/kg) 6, 24 and 48 hours post-surgery.

All animal studies were performed following guidelines from the institutional ethical commission. The experimental design employing non-human primates was approved by the Ethical Committee for Animal Testing of the University of Navarra and by the health department at the government of Navarra (ref: NA-UNAV-02-08).

Viral construction, production and purification

Recombinant AAV vectors were constructed with a transgene cassette encoding the reporter gene Human Herpesvirus 1 strain Thymidine Kinase (HSV1-TK, GenBank

acc# AF243488) or the reporter gene luciferase (GenBank acc# M15077) under the regulation of a chimeric liver specific promoter composed of the human α1-antitrypsin promoter (AAT) with regulatory sequences from the albumin enhancer (Ealb) (Kramer *et al*., 2003). The porphobilinogen deaminase (PBGD) 3' UTR was downstream of the TK or luciferase gene (Kindly provided by Dr A. Fontanellas). The transgene cassette was flanked by AAV2 wild type inverted terminal repeats and the entire cassette was subcloned into the baculoviral transfer plasmid to generate recombinant baculovirus. Pseudotyped AAV2/5 vectors were produced using the Baculovirus Expression Vector System (Protein Sciences Corporation, Meriden, Connecticut USA) as described (Urabe *et al*., 2006). Baculoviral stocks expressing the rAAV genome, VP capsid proteins and Rep proteins were used for AAV production by triple infection of Sf+ insect cells, in a ratio of 1:1:5 .

AAV purification was performed by affinity chromatography using AAV-specific Llama VHH antibodies (Hermens *et al*., 1999; Zolotukhin *et al*., 1999). Viral titres in terms of genome copies (gc) per milliliter (gc/ml) were determined by quantitative polymerase chain reaction (Q-PCR) (Pañeda *et al*., 2009). Capsid protein composition and purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and endotoxin test. Ad-CMV-TK was constructed as described in Peñuelas *et al*. (Peñuelas *et al*., 2005b).

Analysis of TK expression by microPET and PET-CT

PET imaging was performed in a dedicated small animal Philips Mosaic tomograph (Cleveland, Ohio, USA) with 2 mm resolution. The technique is based on the injection of the reporter probe ^{18}F-FHBG prepared as previously described (Peñuelas *et al*., 2005b). For rodents studies, general anaesthesia was induced with 2% isofluorane in 100% O2 gas. Subsequently, the radiotracer was injected via the tail vein (mice: 14.1 ±

7.4 MBq in 100 μL; rats: 37 ± 15 MBq in 200 μL). Animals were immediately awakened for radiotracer uptake and placed back in a cage for sixty minutes. Afterwards, animals were placed prone on the PET scanner bed to perform a static acquisition for 15 minutes under continuous inhalational anaesthesia. In the case of rats, a transmission scan was performed after image acquisition. After PET studies, mice and rats were sacrificed for liver collection and immunohistochemistry (IHC) for TK.

For monkey microPET studies, animals were fasted overnight prior to PET. On the day of the scan, anaesthesia was initially induced with ketamine (10 mg/kg intramuscularly) and maintained throughout the procedure with ketamine/midazolan (10/0.8 mg/kg). After positioning the animal prone in the scanner, a transmission study was carried out for ten minutes. Then, radiotracer with an activity of 75±17 MBq was injected into the saphenous vein and after 60 minutes, a static aquisition of 15 minutes was carried out. In some cases, after microPET studies and in order to obtain additional information about anatomical distribution of ^{18}F-FHBG, monkeys were moved to a hybrid PET/CT tomograph (Biograph DUO, Siemens/CTI, Knosville, TN, USA). CT was performed 1 min before PET scan, with the animal in the same position. CT parameters for a dual-detector helical CT were 130 keV, 50 mAs, 0.8 s per CT rotation, 5 mm slice thickness. A pitch of 1.5. PET images were acquired in 3-D mode with 3 min per bed position. CT images were reconstructed with 2.4 mm increments and PET emission data were corrected for random events, dead time, and attenuation correction and were reconstructed with an OSEM algorithm.

All the microPET images, both for rodents and monkeys, were reconstructed using a three-dimensional RAMLA with a 1 mm voxel size applying dead time, decay, random and scattering corrections, and also attenuation correction for rats and monkeys.

Image quantitative data for the PET are expressed as maximum Standard Uptake Value (SUVmax). To obtain this value, regions of interest (ROIs) were drawn over the liver parenchyma and maximum SUV values were calculated using the formula: tissue activity concentration (Bq/cc)/injected dose (Bq) x body weight (g).

Ex vivo processing of liver biopsies

Liver biopsies were processed for TK immunohistochemistry staining with a primary polyclonal antibody (kindly provided by William Summers, Yale University) using the EnVision TM + System (Dako, Glostrup, Denmark) according to the manufacturers' recommendations and as described (Fontanellas *et al*., 2009).

Total DNA was extracted from liver biopsies using the QIAamp-DNA mini kit from Qiagen in a laminar flow cabinet. Viral DNA was quantified by Q-PCR, using the primers TK-sense (TCCCATGCACGTCTTTATCCT) and TK-antisense (TAAGTTGCAGCAGGGCGTC). Monkey β-actin was quantified using the following primers: sense (CCTGTGGCATCCACGAAAC) and antisense (TTACGGATGTCCACGTCACACT). Rat GADPH was quantified using the following primers: sense (CTTCCACGATGCCAAAGTTC) and antisense (GATGGTGAAGGTCGGTGTG) and mouse GADPH was quantified using the following primers: sense (TGCACCACCAACTGCTTA) and antisense (GGATGCAGGGATGATGTTC). The number of TK copies in each biopsy was normalized with β-actin or GADPH copy numbers. The values shown correspond to the mean copy number from two independent biopsies. Liver samples were stained with H&E for histopathological evaluation.

TK protein expression and purification

The HSV1-TK gene was amplified by PCR from plasmid pSK-TK. The resulting PCR product was directly cloned into the multiple cloning site of pcDNA3.1/V5/His-TOPO

using the Topo cloning kit (Invitrogen) and positive clones were verified by sequencing. The TK gene was subcloned into the pET14b vector (Novagen) that enables the expression of the thymidine kinase protein carrying six histidine residues (His•Tag®) at the N-terminal sequence. The resulting plasmid pET14b-TK were transformed into the BL21 competent *E.coli* (Sigma) for the expression of the recombinant protein. Protein was purified from the supernatant by affinity chromatography (HisTrap; Pharmacia) using a fast protein liquid chromatography platform (AKTA; Pharmacia). The eluted protein was desalted using p10 desalting columns (GE Healthcare) and purified from endotoxins by EndoTrap columns (Profos, Regensburg, Germany). The final protein obtained was quantified by Bradford and analyzed by PAGE and western blot (anti-Histidine antibody and anti-TK antibody).

Analysis of T cell specific immune response in monkeys

To evaluate the cellular immune response induced against the vector or the transgene after rAAV5-EalbAAT-TK vector administration, monkey leukocytes were purified from peripheral blood by centrifugation through ficoll-hypaque (GE Healthcare). Isolated leukocytes ($5x10^5$ cells/mL) were cultured in medium alone, or in the presence of purified rAAV5 capsid proteins (10 µg/ml), or purified recombinant TK protein (10 µg/ml), or PMA 0.05 µg/ml + ionomycin 0.5 µg/ml (PI), in triplicate. After 48 hours cells were harvested, RNA extracted and IFN-γ and GADPH expression was analysed by RT quantitative PCR, using the primers IFN-γ-sense (5′-TGGAGACCATCAAGGAAGACA-3') and IFN-γ -antisense (5′-ACAGTTCAGCCATCACTTGGA-3′) and GADPH sense (5′-GGTCGGAGTCAACGGATTT-3′) and GADPH antisense (5′-CCAGCATCGCCCACTTGA-3′).

Results

TK expression analysis by microPET and IHC in mice

C57BL/6 mice of both genders were injected intravenously with two different doses of rAAV5-EalbAAT-TK vector, $5x10^{12}$ gc/kg and $1x10^{12}$ gc/kg. Three weeks after injection, mice were subject to imaging for TK reporter gene expression using PET, after the administration of ^{18}F-FHBG substrate. After PET analysis, all the animals were sacrificed and liver sections were subject to IHC for TK. As a negative control, two mice received $5x10^{12}$ gc/kg of a rAAV encoding luciferase (rAAV5-EalbAAT-Luc).

Representative microPET imaging results are shown in Figure 1A. Expression of the TK reporter gene was confined to the liver. Wash out of the tracer to the intestines and gall bladder can be detected. The signal intensity was dose-dependent and was consistently stronger in males than in females (also shown in Fig. 1B). Representative images of TK IHC analysis are shown in Figure 1C and the mean values of the percentage of transduction for each group of animals appear in Table 1. The analysis of TK expression by IHC showed a very high correlation between PET SUVmax values and the percentage of transduced hepatocytes ($r^2 = 0.96$; Fig. 1D). Importantly, our data shows that PET technology can detect TK hepatic transduction levels in mice down to 1% efficiency. Of note, no retention of the radioactive tracer in the liver was observed in the mice injected with rAAV5-EalbAAT-luc (Data not shown).

One of the main characteristics of rAAV vectors is their capacity for sustained transgene expression. To monitor long-term expression of TK after rAAV administration, three C57BL/6 female mice were injected with $1x10^{12}$ gc/kg; four and eight weeks later, TK expression was analyzed by microPET. As shown in Table 2, similar SUV maximum values were obtained after the first and second measurements, indicating that TK expression was stably maintained after rAAV injection. After the

second analysis, animals were sacrificed and TK expression was analysed by IHC. Maximal SUV values correlated with the percentage of transduced hepatocytes (data not shown). The same experiment was performed in C57BL/6 male mice obtaining similar results (data not shown).

TK and luciferases expression analysis in rats

Four male and four female Sprague-Dawley rats were injected intravenously, via the tail vein, with a dose of $1x10^{12}$ gc/kg of rAAV5-EalbAAT-TK. One male and one female rat were injected with the corresponding dose of rAAV5-EalbAAT-Luc (negative control) and as a positive control one male was injected with adenoviral vector encoding TK under a cytomegalovirus promoter, Ad-CMV-TK, at a dose of 5×10^{11} pfu/kg. TK expression was analyzed by microPET one week and three weeks after AAV injection and two days after adenovirus injection. Representative microPET imaging results are shown in Figure 2A. A strong signal was detected in the animals injected with the adenovirus expressing TK, while a very faint signal was detected in male rats injected with rAAV5-EalbAAT-TK. Female rats injected with rAAV5-EalbAAT-TK or rAAV5-EalbAAT-luciferase demonstrated similar levels of radiotracer retention. Similar results were obtained one and three weeks after AAV injection. These results indicate that at this dose rAAV5 does not transduce the liver of female rats (Fig. 2A). IHC corroborated the absence of liver transduction in female rats and a very low transduction (< 0.15 %) in male rats. A large proportion of hepatocytes were transduced in rats administered with adenovirus expressing TK (29%; Fig. 2B). To confirm the lower transduction efficiency of AAV5 in Sprague-Dawley rats the presence of viral genomes in the liver was quantified by quantitative PCR in rats and in C57BL/6 mice injected with the same dose of virus. As shown in fig 2C, the number of genomes in the liver correlate with TK expression levels. In order to determine if the lower transduction level was due to the

development of an immune response against the virus or the transgene, lymphocytes obtained form rat peripheral blood 21 days after vector injection were stimulated with recombinant TK protein or AAV5 empty capsids, as positive control lymphocytes were stimulated with concanavalin A. 24 hours after stimulation lymphocytes were harvested, RNA extracted and IFN-γ expression was analysed by qPCR. No IFN-γ was detected after stimulation with AAV5 capsid or recombinant protein (data not shown). To further confirm the lower transduction level mediated by AAV serotype 5 in Sprague Dawley rats in comparison to C57BL/6 mice, 4 animals of each specie were injected with a dose of 2.5 x 10^{12} vg/Kg of an AAV5 virus expressing luciferase (rAAV5-EalbAAT-Luc) and transgene expression was followed for one month. As shown in figure 2D, luciferase expression was sustained over time in both groups, but luciferase expression was 10 times higher in C57BL/6 mice.

TK expression analysis by microPET and IHC in non-human primates

Two male and two female monkeys were injected with rAAV5-EalbAAT-TK by intravenous injection. One male (male low dose, MLD) and one female (female low dose, FLD) received a low dose of 1x10^{12} gc/kg, and one male (male high dose, MHD) and one female (female high dose, FHD) received a high dose of 2.5x10^{12} gc/kg. rAAV5 vector delivery led to no untoward clinical, haematological or serum chemistry responses in macaques (Pañeda *et al*. manuscript in preparation). TK expression analyses by microPET were performed prior to and 15, 30 and 60 days after rAAV5-EalbAAT-TK injection following ^{18}F-FHBG substrate administration. Liver biopsies were taken 30 and 70 days after virus injection. All the animals were sacrificed 3 months after vector injection.

Figure 3A illustrates microPET imaging from the studies carried out in monkeys before (basal studies) and 15 days post-injection of the vector. Specific retention of the

radiotracer due to TK activity is confined to the liver. A strong signal can also be detected in the intestines and gall bladder due to the metabolic wash out of the radiotracer. MicroPET analysis was repeated 30 and 60 days after vector injection. Quantitative analysis of radioactive tracer retention in the liver was estimated by region of interest (ROI) measurement in the liver using static microPET images and expressed as SUVmax. As shown in Figure 3B, radiotracer incorporation was maximal 15 days after vector administration and correlates with the dose of rAAV5-EalbAAT-TK injected. SUV max values were higher in males than in females at the same dose, as has been previously described in rodents (Pañeda *et al*., 2009). However, contrary to what we observed in mice, expression was not maintained in monkeys over time, where, after 30 days TK expression decreases and returns to background levels a month later (60 days). TK expression was analysed by IHC in liver biopsies and in the whole liver after sacrifice. Very low or no TK expression was detected in liver biopsies as shown in Figure 3C, 0.039 % in MLD; 0.59 % in FLD and 0.79 % in FHD and 0 in MHD. Surprisingly, the transduction efficiency was higher in females than in males. In fact, there is an inverse correlation between the TK expression level detected by PET and by IHQ. A possible explanation for this finding is that the more robust transgene expressions observed in male monkeys induce a stronger immune response resulting in a faster disappearance of the transgen. The analysis of the biopsy obtained 70 days after vector injection, or at sacrifice, showed no TK expression in all the animals, confirming the result obtained by microPET. Analysis of viral DNA in liver biopsies by quantitative PCR revealed a disappearance of vector genomes from the liver (Fig. 3D). All together, the data suggests that rAAV5-EalbAAT-TK-transduced hepatocytes were eliminated, most likely due to an immune response that appears to have developed faster in male monkeys.

PET-CT data in non-human primates

All monkeys were analyzed in a clinical PET-CT scanner 15 days after rAAV5-EalbAAT-TK administration and two hours after performing the microPET study. This study, performed 3 hours after radiotracer injection, should provide detailed information about the biodistribution of TK expression, since a whole body CT-scan is superimposed over the PET image. Figure 4, illustrates PET-CT images of male and female monkeys receiving the high doses. For both genders, the radioactive tracer was detected in the liver by virtue of rAAV-delivered TK (and in intestines and bladder due to the wash out of the radiotracer).

Analysis of immune response against TK and AAV5 capsid proteins

The disappearance of TK expression from the livers of rAAV5-EalbAAT-TK-injected monkeys suggest an immune response against the recombinant vector, where the non-self proteins are the recombinant TK protein and the viral capsid proteins. Before and every month after viral administration, lymphocytes were obtained from each monkey. At the end of the study, the lymphocytes were incubated with purified rAAV5 capsid proteins, purified recombinant TK protein, PMA + ionomycin (P/I) or, medium alone. 48 hours after incubation with the different stimuli, cells were harvested and IFN- γ and GADPH expression was analysed by Q-PCR. No IFN-γ production was detected before vector administration after stimulation with recombinat TK protein of viral capsid protein, but were fully activated after P/I stimulation except for MLD monkey that for an unknown reason, did not respond (data not shown). However, as shown in Figure 5A, IFN-γ production was detected after incubation with the recombinant TK protein but not with AAV5-capsid proteins in lymphocytes obtained from the four monkeys. Interestingly, the immune response was stronger and can be detected for a longer period of time in the male than in female monkeys. In order to determine if the immune

response results in a toxic effect complete serum biochemistry analysis were performed 7, 15, 30, 60, and 90 days after viral injection. Urea, bilirrubin, ALP, AST, ALT, total protein, albumin, GGT, creatinine, Na, Cl, K, glucose, cholesterol, calcium and CPK concentration in serum were determined. All the values were in the normal range except for AST which was elevated at day 7 and 15 after vector injection in MHD monkey, and at day 30 in the rest of the animals (Fig. 5B). Thus, an immune response against the transgene was deemed more likely responsible for the disappearance of transduced hepatocytes.

Discussion

Recombinant AAV vectors are currently among the most frequently used viral vectors for gene therapy (Grieger and Samulski, 2005; Daya and Berns, 2008; Luebke *et al*., 2009; Stieger *et al*., 2009; Terzi *et al*., 2008; Markakis *et al*., 2010; Alexander *et al*., 2008). Since human populations are natural hosts for AAV viruses one of the limitations for the clinical use of rAAV is the presence of anti-AAV neutralizing antibodies The use of different pseudotyped AAV capsids (serotypes) has allowed for broad tissue tropisms and to avoid pre-existing immunity against AAV (Markakis *et al*., 2010; Boutin *et al*., 2010). As the prevalence of neutralizing antibodies against AAV-5 and -8 is lower in humans it is likely that these serotypes may be useful in patients with high-titre neutralizing anti-AAV-2 antibodies (Boutin *et al*., 2010).

Molecular imaging plays a major role in the field of gene therapy, allowing quantitative monitoring of the magnitude of gene expression, as well as analysing the location and duration over time (Gambhir *et al*., 1999; Peñuelas *et al*., 2002; Peñuelas *et al*., 2005a; Peñuelas *et al*., 2005b; Fontanellas *et al*., 2009). Radionuclide imaging methods such as PET have excellent depth sensitivity and can detect accumulation of gene expression within vector transduced organs anywhere in the body. When combined with other forms of tomographic imaging, such as CT, fusion images of functional and anatomic data provide more detailed *in situ* information of reporter gene expression and localization. In this study, rAAV 5 expressing herpes simplex virus thymidine kinase under the control of a liver specific promoter (rAAV5-EalbAAT-TK) was used to monitor rAAV5-mediated gene expression and duration in non-human primates.

First, we tested the sensitivity and robustness of this technique in rodent models. We found a direct correlation between SUVmax values obtained after PET analysis of rAAV5-EalbAAT-TK injected mice with the liver transduction efficiency calculated by

immunohistochemistry as TK-positive cells ($r^2 = 0.957$). Our results are in agreement with previous studies performed by Liang and co-workers (Liang *et al*., 2002), where they reported a linear correlation between hepatic FHBG retention and HSV-TK activity in liver extracts of mice injected with adenovirus encoding TK ($r^2 = 0.96$) (Liang *et al*., 2002). Since rAAV vectors have been reported to mediate long-term expression in rodents and non-human primates, it was considered important to follow TK expression over time, non-invasively, by PET imaging. Our results indicate that in rodents TK expression is maintained for at least two months after vector injection. We found dramatic differences in liver transduction depending not only by the gender, as previously reported, but also depending on the species. After a single injection of $1x10^{12}$ gc/kg in C57BL/6 male mice, 3% of the hepatocytes were transduced, while only 0.05% of the hepatocytes from Sprague-Dawley male rats showed TK expression. Despite the low transduction efficiency of hepatocytes, TK activity can be detected and quantified by microPET in these rats, demonstrating the high sensitivity of this technique to detect gene expression in living subjects.

rAAV-mediated gene transfer studies in non-human primates are not very abundant (Nathwani et al., 2002; Nathwani *et al.*, 2006; Davidoff *et al.*, 2005; Gao *et al*., 2006a; Gao *et al*., 2006b; Jiang *et al*., 2006; Nathwani *et al*., 2007). In rhesus macaques, injection of rAAV serotypes 2, 5 and 8 expressing Factor IX, rhCG or Epo resulted in long term expression of the transgene (Gao *et al*., 2006b; Jiang *et al*., 2006; Nathwani *et al*., 2007). In our study, four monkeys were injected with two different doses of rAAV5-EalbAAT-TK and were analysed at days 15, 30, and 60 after viral administration by microPET after ^{18}F-FHBG administration. We found that radiotracer incorporation can be detected in the livers of rAAV5-EalbAAT-TK injected monkeys 15 days after vector injection. PET-CT analysis showed that TK expression was

restricted to the liver. The signal intensity depends on the dose of virus administered and on the gender of the monkey. Interestingly, the intensity of radiotracer incorporation was higher in male than in female monkeys as previously shown in rodent models. This observation is particularly important, since macaques are more likely to reflect the transduction efficacy of AAV vectors in humans than murine or other small animals. Thus, differences dependent on the gender would be also expected in humans and should be taken into account when designing clinical trials.

Long term analysis showed that, contrary to the long term expression of human factor IX or hCG observed in NHPs (Nathwani *et al*., 2002; Nathwani *et al.*, 2006; Davidoff *et al.*, 2005; Gao *et al*., 2006a; Gao *et al*., 2006b; Jiang *et al*., 2006; Nathwani *et al*., 2007), expression of TK disappeared from the livers of monkeys injected with rAAV5-EalbAAT-TK. PET analysis performed at day 30 and 60 after viral injection showed a significant decrease or disappearance of radiotracer signal from the liver, compared to day 15. Immunohistochemistry, performed at day 21, confirmed the disappearance of TK expression in the male monkeys that received the highest dose of rAAV5 vector and also showed very low expression in the rest of the monkeys. Analysis of liver biopsies obtained at day 60 and from different liver lobes (upon sacrifice) revealed the disappearance of TK expression from all treated monkeys. This was corroborated by the loss of vector genomes.

Interestingly, the expression levels at day 21 were lower in males than in female monkeys, in contradiction with the microPET data. Since we have observed a direct correlation between TK expression analysed by IHC and by PET in rodents, our explanation for the discrepancy to NHPs is that the factor responsible for the disappearance of TK-transduced cells developed faster in males than in females.

These results suggest that the administration of the rAAV5-EalbAAT-TK virus has induced the development of a specific immune response that eliminated the transduced hepatocytes. Since the only foreign components of the virus are the transgene and the capsid proteins, we have analysed the production of IFN-γ by the lymphocytes after stimulation with rAAV5 capsid proteins and recombinant TK protein. We have only observed IFN-γ production after incubation with TK, indicating that an immune response against the transgene was developed. The more robust transgene expression in male monkeys would likely make TK more immunogenic explaining why gene expression is more rapidly lost in males. Furthermore, analysis of transaminase levels in serum showed a transient elevation of AST indicating a certain degree of liver injury that developed in the male monkey receiving the highest dose (MHD). Our results correlate with those described by Gao and co-workers where primates elicited vibrant cytotoxic T cell responses to GFP that correlated with hepatitis and loss of transgene expression (Gao *et al*., 2003; Wang *et al*., 2010). These studies indicate, that under some conditions, primates may activate more robust T cell responses to transgene products than mice. Furthermore, Mercier-Letondal showed that the administration of HSV1-TK-expressing, gene-modified T cells to patients induces a T cell response preferentially targeting TK that eliminate the transduced cells (Mercier-Letondal *et al*., 2008).

In summary, PET technology is a non-invasive method with a high value to analyze vector transduction with the reporter gene thymidine kinase in mice and rats. The quantification value by SUVmax is correlated with the transduction efficiency estimated by IHC. TK expression can be detected by microPET and PET-CT in liver monkeys after AAV5-mediated gene transfer. However, due to the immunogenicity of the transgene, TK expression was only detected transiently. This model could be useful for

the development of new strategies to circumvent immune response against the transgene in NHP.

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Legends

FIG. 1. *In vivo* and *ex vivo* analysis of the expression of the HSV-TK reporter gene in C57BL/6 female and male mice following injection through the tail vein of rAAV5-EalbAAT-TK. (**A**) MicroPET scans of a representative male and a female mice three weeks after injection with $5x10^{12}$ gc/Kg rAAV5-EalbAAT-TK, following ^{18}F-FHBG injection. (**B**) Hepatic ^{18}F-FHBG retention measured by microPET in male and female mice injected with $5x10^{12}$ gc/Kg or $1x10^{12}$ gc/kg of rAAV5-EalbAAT-TK and expressed as SUV maximum values. (**C**) Representative images of IHC analysis of TK expression in the livers of male and female mice injected with $5x10^{12}$ gc/kg or $1x10^{12}$ gc/kg of rAAV5-EalbAAT-TK. The number of TK expressing cells was quantified from more than 50 fields of each liver and using the Image J program, transduction rates were calculated. (**D**) Correlation between TK expression analysis calculated by ^{18}F-FHBG retention (SUVmax) and by IHC for each mice ($r^2 = 0.9571$).

FIG. 2. *In vivo* analysis of the expression of the HSV-TK reporter gene in Sprague-Dawley rats following injection through the tail vein of Ad-CMV-TK, rAAV5-EalbAAT-TK, rAAV5-EalbAAT-luc. (**A**) MicroPET scan of a male rat two days after intravenous injection of 5×10^{11} pfu/kg Ad-CMV-TK; a male rat three weeks after injection with $1x10^{12}$ gc/kg rAAV5-EalbAAT-luc, a male rat three weeks after injection with $1x10^{12}$ gc/kg of rAAV5-EalbAAT-TK, and a female rat three weeks after injection with $1x10^{12}$ gc/g of rAAV5-EalbAAT-TK, following ^{18}F-FHBG injection. Hepatic FHBG retention measured by microPET is expressed as SUV maximum values. (**B**) Representative images of the IHC analysis of TK expression in the liver of male rats mice injected with $1x10^{11}$ gc/kg Ad-CMV-TK or $1x10^{12}$ gc/kg of rAAV5-EalbAAT-TK. (**C**) Viral copies per diploid genome in the liver of male and female mice and rats

were quantified by quantitative PCR 21 days after the injection of $1x10^{12}$ vg/Kg of rAAV5-EalbAAT-TK. data are shown as means ± SD. (**D**) Female C57BL/6 mice (n = 4) and female Sprague-Dawley rats (n = 4) were injected intravenously with 2.5 x 10^{12} vg/Kg of rAAV5-EalbAAT-Luc. Seven, 15 and 30 days after vector injection luciferase expression was quantified tracing regions of interest in the liver area and light intensity was quantified using photons/sec, data are shown as means ± SD.

FIG. 3. *In vivo* analysis of the expression of the HSV-TK reporter gene in *Macaca fascicularis* monkeys following injection of rAAV5-EalbAAT-TK into the saphenous vein. (**A**) Reconstructed images of the microPET study of the four monkeys included in the study injected with $1x10^{12}$ gc/kg or $2.5x10^{12}$ gc/kg before and 15 days after rAAV5-EalbAAT-TK and 1h after administration of the radiotracer. Liver (lv), gall bladder (gb), intestine (i), bladder (b). (**B**) Hepatic ^{18}F-FHBG retention measured by microPET in male and female monkeys injected with $1x10^{12}$ gc/kg or $2.5x10^{12}$ gc/kg of rAAV5-EalbAAT-TK and expressed as SUV maximum values at basal, and 15, 30 and 60 days after vector injection. [*Due to a technical problem SUVmax values for monkeys MHD and FHD at day 30 could not be calculated] (**C**) Representative images of the IHC analysis of TK expression in liver biopsies obtained 21 days after vector injection. (**D**) Total DNA was isolated from liver biopsies obtained from four rAAV5-EalbAAT-TK-injected and two uninjected monkeys. Vector genome copies were determined using a Q-PCR specific to HSV-TK, normalised to β–actin copy numbers. Values corresponding to the mean copy number from two independent liver biopsies.

FIG. 4. PET-CT image of male and female monkey 15 days after injection with $2.5x10^{12}$ gc/kg rAAV5-EalbAAT-TK; and two hours after administration of the radioactive tracer. Liver (lv), bladder (b).

FIG. 5. Analysis of TK and AAV5 capsid immune response and transaminase levels is serum. (**A**) Peripheral blood lymphocytes were obtained from all the monkeys before and every month after vector injection. $2x10^5$ lymphocytes were incubated for 48 hours with medium alone, 10 μg/ml of purified recombinant TK protein, 10 μg/ml of AAV5 capsid proteins, or PMA 0.05 μg/ml + ionomycin 0.5 μg/ml, in triplicate. The lymphocytes were harvested and RNA extracted to determine IFN-γ and (house-keeping) GADPH expression by Q-RT-PCR. (**B**) AST levels were measured in serum before and 15, 30, and 60 days after viral injection. The horizontal dashed lines represent the upper and lower AST normal values in *macaca fascicularis*; the information was obtained from the Canadian Council on Animal Care (CCAC) and from California National Primate Research Center

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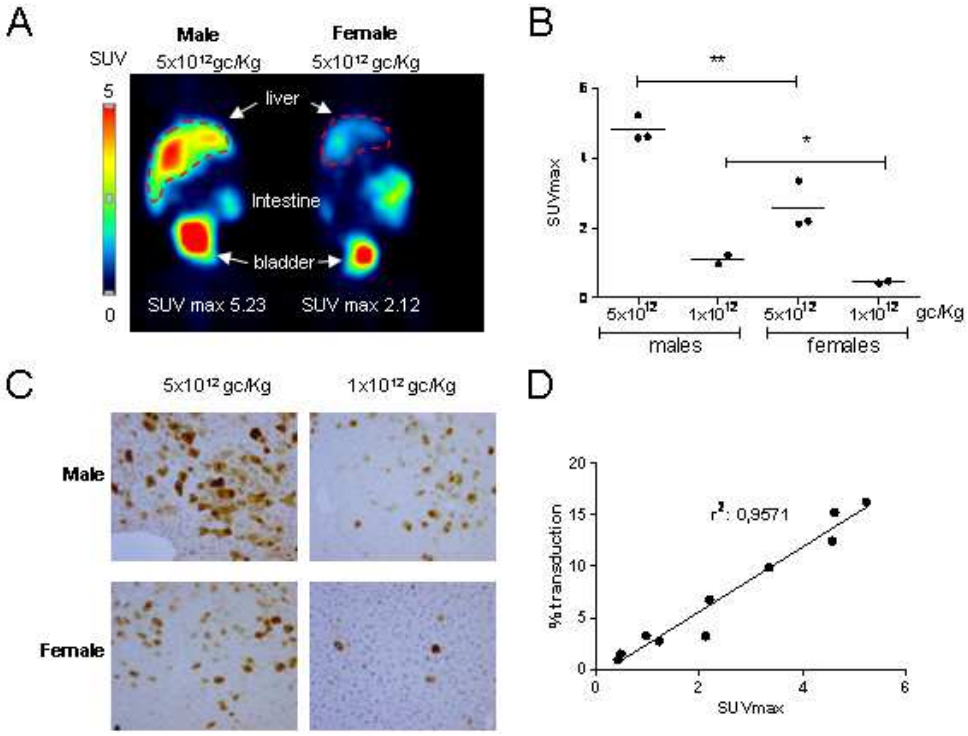

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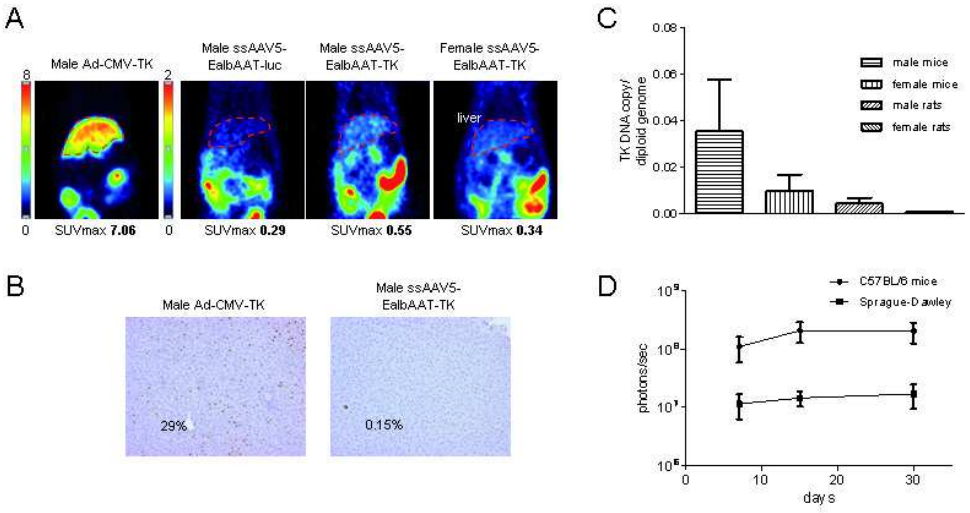

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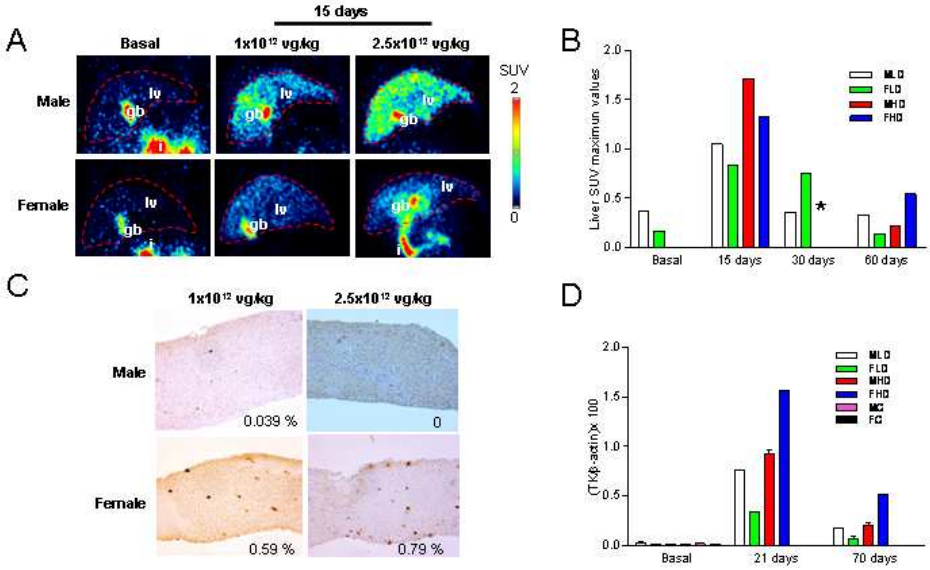

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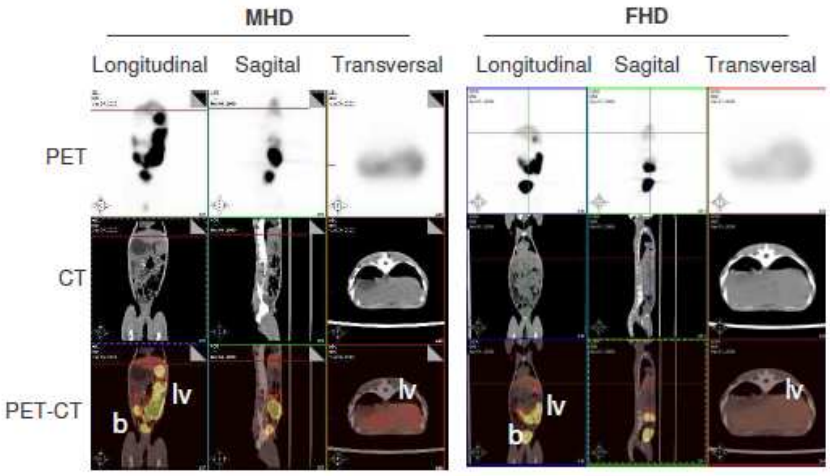

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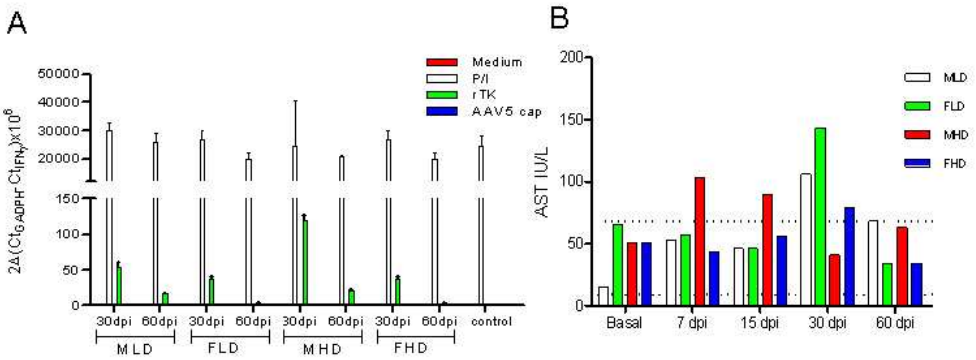

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Table 1: SUVmax values and hepatocytes transduction rate in male and female C57BL/6 mice three weeks after injection of two different doses of ssAAV5-EalbAAT-TK

Sex	Virus	Dose	SUVmax	Transduction rate
Male	ssAAV5 - EalbAAT -tk	5 x 10^{12} gc/kg	4,62 +/- 0,36 %	38,4 +/- 6,2 %
Male	ssAAV5 - EalbAAT -tk	1 x 10^{12} gc/kg	1,10 +/- 0,18 %	8,35 +/- 3,03 %
Female	ssAAV5 - EalbAAT -tk	5 x 10^{12} gc/kg	2,19 +/- 0,69 %	9,72 +/- 1,12 %
Female	ssAAV5 - EalbAAT -tk	1 x 10^{12} gc/kg	0,45 +/- 0,04 %	0,89 +/- 0,59 %

Table 2: SUVmax values of C57BL/6 female mice four weeks (SUV1) and eight weeks (SUV2) after injection of $1x10^{12}$ gc/Kg of ssAAV5-EalbAAT-TK

	SUV1	SUV2
TK22	0,6	0,66
TK23	0,53	0,71
TK24	0,72	0,75