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Intrahepatic Injection of Recombinant Adeno-Associated Virus Serotype 2 Overcomes Gender-Related Differences in Liver Transduction

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ABSTRACT

The liver is an attractive organ for gene therapy because of its important role in many inherited and acquired diseases. Recombinant adeno-associated viruses (rAAVs) have been shown to be good candidates for liver gene delivery, leading to long-term gene expression. We evaluated the influence of the route of administration on rAAV-mediated liver transduction by comparing levels of luciferase expression in the livers of male and female mice after injection of rAAV serotype 2, using three different routes of administration: intravenous (IV), intraportal (IP), or direct intrahepatic (IH) injection. To determine transgene expression we used a noninvasive optical bioluminescence imaging system that allowed long-term *in vivo* analysis. After IV injection dramatic differences in liver transgene expression were observed, depending on gender. When IP injection was used the differences were reduced although they were still significant. Interestingly, direct intrahepatic injection of rAAV vectors was associated with the fastest and strongest onset of luciferase expression. Moreover, no gender differences in liver transduction were observed and luciferase expression was confined to the site of injection. Thus, direct intrahepatic injection of rAAV offers specific advantages, which support the potential of this route of administration for future clinical applications.

INTRODUCTION

ADENO-ASSOCIATED VIRUSES are small, single-stranded DNA viruses derived from a replication-deficient member of the parvovirus family and do not appear to cause any disease in humans (Lai *et al*., 2002). Recombinant adeno-associated vectors (rAAVs) are safe and effective gene therapy vectors. They are not pathogenic, do not elicit significant immune responses, transduce both quiescent and dividing cells, and can provide high-level and long-term transgene expression (Lai *et al*., 2002; Bessis *et al*., 2004; Buning *et al*., 2004). Furthermore, it has been described that rAAV integration is inefficient and the persistence of rAAV genomes in tissues is largely due to episomal genomes (Nakai *et al*., 2001; McCarty *et al*., 2004). Many of their biological properties make AAV an attractive gene transfer system for clinical application.

AAV particles enter the cell via receptor-mediated endocytosis through clathrin-coated pits. Inside the cells and after endosomal acidification, the virus is released into the cytosol. Upon release into the cytosol, it accumulates perinuclearly and slowly penetrates into the nucleus. Once in the nucleus, the transfection efficiency is further limited by the need to convert the single-stranded (ss) genome into double-stranded (ds), transcriptionally active forms (Bartlett *et al*., 2000). Depending on the cell type, dsDNA conversion occurs by *de novo* synthesis of the second strand or through annealing of complementary plus and minus single-stranded molecules (Nakai *et al*., 2000).

Recombinant AAVs show some degree of liver specificity after systemic injection (Summerford *et al*., 1999), and have been proved to be highly effective vehicles for gene therapy in several disorders such as glycogen storage disease type II (Sun *et al*., 2005), hemophilia (High, 2002; Wang *et al*., 2005),

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phenylketonuria (PKU) (Mochizuki *et al*., 2004), and hypercholesterolemia (Lebherz *et al*., 2004). They have been successfully used to transduce factor IX to mouse, dog, and nonhuman primate livers and have achieved persistent, curative concentrations of the functionally active molecule (Snyder *et al*., 1997; Summerford *et al*., 1999; Harding *et al*., 2004; Wang *et al*., 2005). rAAV vectors are currently being evaluated in patients with hemophilia (High, 2004). The routes of administration for rAAV most frequently used to transduce the liver are intravenous injection and portal vein infusion (Harding *et al*., 2004). AAV administration by hepatic arterial infusion resulted in highly efficient liver transduction (Ohashi *et al*., 2005). In mice, injection into the splenic capsule has been used to efficiently deliver the AAV vector to the portal circulation (Daly, 2004). Direct intrahepatic injection has been used in fetuses (Mitchell *et al*., 2000) and in adult female mice (Snyder *et al*., 1997). Intrahepatic injection of an AAV expressing factor IX resulted in high levels of protein expression; however, liver transduction by this route has not been characterized (Snyder *et al*., 1997).

Striking differences in liver transduction have been reported after portal vein injection of rAAV serotypes 2 and 5, depending on the sex of the animal (Davidoff *et al*., 2003). Furthermore, in a mouse model of PKU, about three times more rAAV must be injected into female mice than into male mice to achieve an equivalent reduction in serum phenylalanine (Mochizuki *et al*., 2004). These data have been further corroborated by our results in woodchucks chronically infected with the woodchuck hepatitis virus, in which the intraportal administration of rAAV carrying the woodchuck interferon-α (IFN-α) gene resulted in dramatic differences in cytokine expression depending on the sex (Berraondo *et al*., 2005). This would seem to indicate that sex differences in AAV-mediated liver transduction found in mice are not species specific and may represent a problem in clinical applications. Analysis of transgene expression in the rodent brain after AAV injection into the thalamus indicates that sex also influences AAV transduction efficiency in this organ (Dodge *et al*., 2005).

The number of hepatocytes that are stably transduced, even when high doses of rAAV vector are delivered, is relatively low (Nakai *et al*., 2002; Sanlioglu *et al*., 2004; Zhong *et al*., 2004). This might represent a problem when a large animal model is used or when a gene therapy protocol is to be applied in a clinical trial, because of the large amounts of recombinant vector that would have to be administered. Therefore, we investigated whether intrahepatic injection of rAAV might be superior to other injection methods for safe and effective transduction of the liver. Three different routes of administration targeting the liver were used: intrahepatic (IH), intraportal (IP), and intravenous (IV, via the tail vein) injection. Levels of luciferase expression were then compared. The use of a noninvasive optical bioluminescence imaging system allowed long-term quantitative evaluation of transgene expression in living animals as well as the study of vector biodistribution. During these analyses we observed significant differences in AAV-mediated liver transgene expression depending on the route of administration and the gender of the animal. Direct intrahepatic injection of the virus resulted in faster, stronger, and localized liver transduction and led to transgene expression levels in females comparable to those in males injected by the IV or IP route.

MATERIALS AND METHODS

rAAV vector construction

Construction of AAV-Luc was performed as described (Berraondo *et al*., 2005). Briefly, a fragment containing the luciferase cDNA was obtained by polymerase chain reaction (PCR) from plasmid pCDNA2.1-Luc. Plasmid pGTC-wIFN-WRE was digested with *Eco*RI to obtain the expression cassette without wIFN and was treated with T4 DNA polymerase to create blunt ends. Both fragments were ligated to obtain the plasmid pGTC-Luc-WRE. The expression cassette from this plasmid was obtained by digestion with *Spe*I and *Sal*I and cloned into the plasmid pAAV-Luc. This plasmid was digested with *Xba*I and *Sal*I and both fragments were ligated. This plasmid was transformed into *Escherichia coli* strain SURE (Stratagene, La Jolla, CA) and the integrity of viral inverted terminal repeats (ITRs) was confirmed by digestion with *Pvu*II, *Sma*I, and *Sna*BI.

rAAV vector production and purification

Plasmid with the expression cassette flanked with ITRs and helper plasmid pDG (Zhong *et al*., 2004) were prepared with an EndoFree plasmid mega kit (Qiagen, Valencia, CA). A mixture of plasmid (20 μg of AAV plasmid and 55 μg of pDG per plate) was transfected into 293T cells, using linear polyethylenimine (25 kDa; Polysciences, Warrington, PA) as described in Durocher *et al*. (2002). After 48 hr cells were harvested, resuspended in Dulbecco's modified Eagle's medium (DMEM; 2.5 ml/plate), and lysed by two freeze–thaw cycles. Virus was digested with 0.1 mg each of DNase and RNase (both from Roche Diagnostics, Mannheim, Germany) for 30 min at 37°C and centrifuged for 15 min at 4°C at 3000 rpm. The supernatant was treated with 0.5% deoxycholic acid (Sigma, St. Louis, MO) for 30 min at 37°C. It was then clarified by filtration through a 0.22-μm filter and the virus was loaded into a HiTrap heparin column (Amersham Biosciences, Uppsala, Sweden) that had been equilibrated with phosphate-buffered saline (PBS, pH 7.4; GIBCO, Paisley, UK) at 1 ml/min. The matrix was washed with 25 ml of PBS–0.1 *M* NaCl and virus was eluted with 15 ml of PBS with 0.4 *M* NaCl. To exchange the buffer and to concentrate the virus, it was applied to an ULTRAFREE-15 centrifugal filter device (Millipore, Bedford, MA), using PBS-MK (PBS containing 1 m*M* $MgCl_2$ and 2.5 m*M* KCl).

For titration an aliquot was digested with DNase and treated with proteinase K (Roche Diagnostics). After inactivation, the titer was determined by real-time PCR. Primers and TaqMan probes (Applied Biosystems, Foster City, CA) were designed to amplify a fragment of the luciferase gene, using Primer Express software (Applied Biosystems). Primers and probe were as follows: sense, 5′-AACATAAAGAAAGGCCCGGC-3′; antisense, 5′-GCCTTATGCAGTTGCTCTCCA-3′; probe, 5′-CATTCTATCCGCTGGAAGATGGAACCG-3′.

The reaction was performed in 20 μl with 2 μl of sample, 2 μl of reaction mix (LightCycler FastStart DNA Master hybridization probes; Roche Diagnostics, Basel, Switzerland), 3.2 μl of $MgCl_2$ (25 m*M*), 0.6 μl of each primer (10 m*M*), and 0.4 μl of probe (5 m*M*). PCR was performed according to the following parameters: 10 min at 95°C (denaturation) and 40 cy-

cles of 0.1 sec at 95°C and 20 sec at 60°C (hybridization/elongation). The fluorescence signal delivered during PCR amplification was monitored with the LightCycler system (Roche Diagnostics). The titer was expressed as viral genomes (VG) per milliliter.

Mice and AAV administration

BALB/c or C57BL/6 mice were obtained from Harlan (Barcelona, Spain), housed under specific pathogen-free conditions, and handled according to the guidelines of our institution (Centro de Investigación Farmacobiológica Aplicada, Pamplona, Spain).

AAV administration was performed in 8-week-old mice. For intrahepatic and intraportal injection, mice were anaesthetized with ketamine–xylazine and a partial laparotomy was performed. Mice received a direct injection of 50 μl of rAAV virus into the left medial lobe of the liver or into the spleen. Intravenous injection was performed by injecting a total volume of 200 μl through the tail vein.

Bioluminescence measurement in living mice

Mice were anesthetized with ketamine–xylazine and 100 μl of D-luciferin (Xenogen, Alameda, CA) at a concentration of 30 mg/ml, diluted in 150 m*M* NaCl solution, was injected by

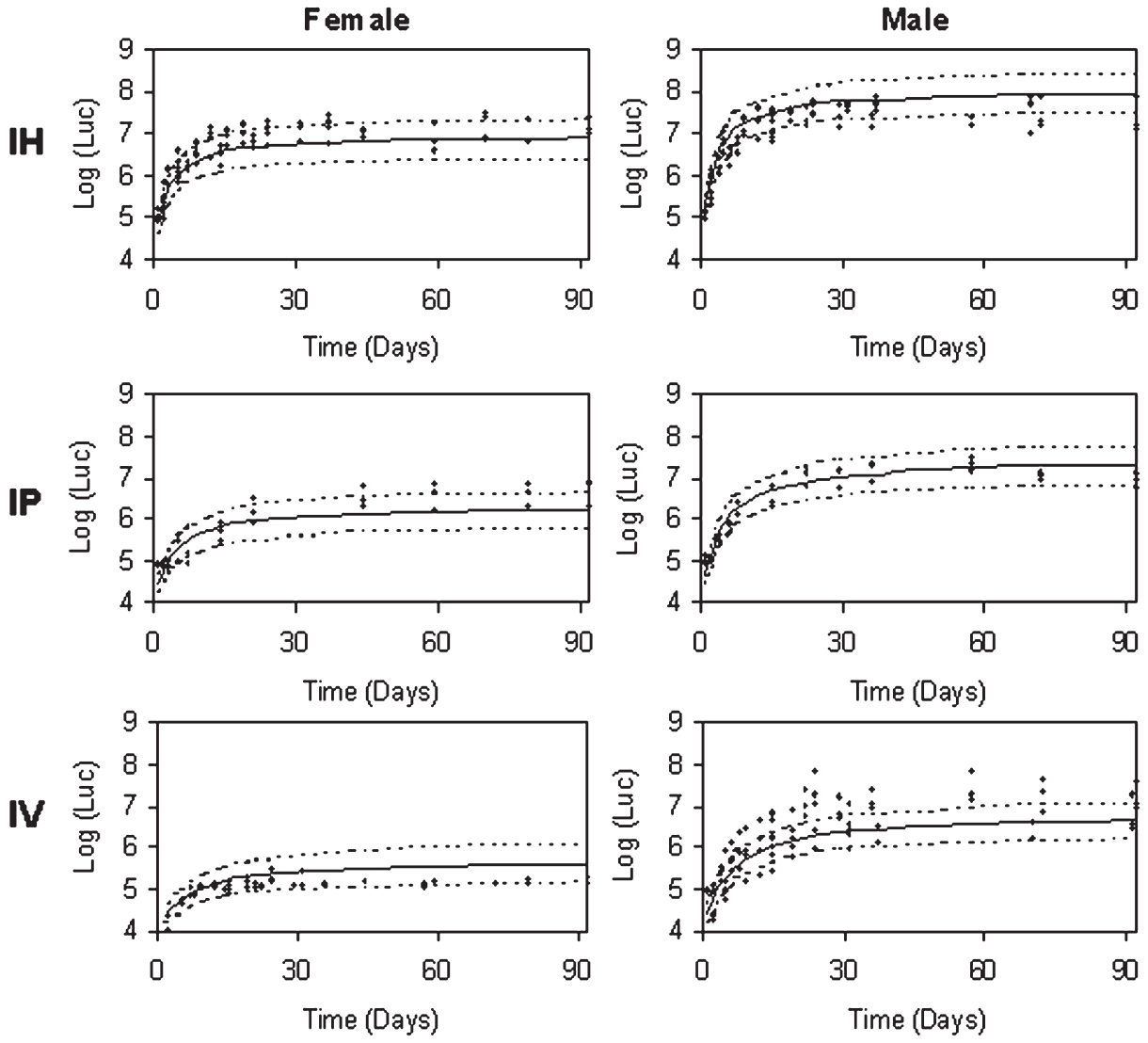

FIG. 1. Short-term *in vivo* CCD signal intensity in the liver of BALB/c mice injected with low-dose AAV-Luc. Male and female BALB/c mice were injected with AAV serotype 2 expressing luciferase gene, at a dose of 2.5×10^{10} genome-containing particles. AAV-Luc was injected intravenously (IV; $n = 8$ males and 8 females), intraportally (IP; $n = 3$ males and 3 females), or intrahepatically (IH; $n = 8$ males and 8 females). Subsequent imaging was performed for luciferase expression, using D-luciferin injected intraperitoneally (150 mg/kg) and starting on day 1 and every week thereafter. Total bioluminescence was quantified and plotted over time. Each dot represents the decimal logarithm of bioluminescence for each animal at a time point. A solid line shows the mean value of each group; dashed lines indicate the confidence interval.

TABLE 1. L_{max} AND T_{50} VALUES FROM BALB/c MICE TRANSFER WITH 2.5×10^{10} VG OF AAV-Luc

	Female			*Male*		
	IV [M (SD)]	*IP [M (SD)]*	*IH [M (SD)]*	*IV [M (SD)]*	*IP [M (SD)]*	*IH [M (SD)]*
L_{max}	1.46 (0.13)	2.64 (0.32)	3.26 (0.23)	3.21 (0.53)	3.38 (0.13)	3.80 (0.24)
T_{50} (days)	5.92 (0.03)	4.35 (0.13)	2.39 (0.24)	5.95 (0.18)	4.14 (0.10)	2.35 (0.21)

Abbreviations: L_{max}, maximum increase in bioluminescence from baseline; M, mean value; SD, standard deviation; T_{50}, time necessary to reach the bioluminescence value $L_{max}/2$.

the intraperitoneal route. The animals were placed in the imaging chamber of the Xenogen IVIS system (Xenogen), which includes a cooled charge-coupled device (CCD) camera. A gray-scale photograph of the animals was acquired, followed by bioluminescence acquisition. Regions of interest (ROIs) were drawn over the positions of greatest signal intensity on each animal, as well as over regions of "no" signal, which were uses as background readings. Light intensity was quantified as photons/sec/cm^2/sr. The gray-scale photograph and data images from all studies were superimposed, using Living Image (Xenogen).

For the *ex vivo* analysis of luciferase expression, animals were killed on day 92 after AAV-Luc injection and various organs were collected and frozen in liquid nitrogen. The organs collected were as follows: testicles or ovaries, spleen, stomach, lungs, heart, kidney, and liver (separated into left medial lobe, right medial lobe, and right lateral lobe). Tissue samples were homogenized in 1× lysis buffer and the protocol to detect luciferase in cells (Promega, Madison, WI) was followed.

Data analysis

All data were analyzed with nonlinear mixed effect models, using MONOLIX software (http://www.math.u-psud.fr/~lavielle/monolix/). Selection between models was based on the precision of the estimated parameters, goodness-of-fit plots, and the value of the Akaike information criterion (AIC). The model with the lowest AIC value was selected, because it indicates that precision of model parameters and data description was adequate (Ludden *et al*., 1994).

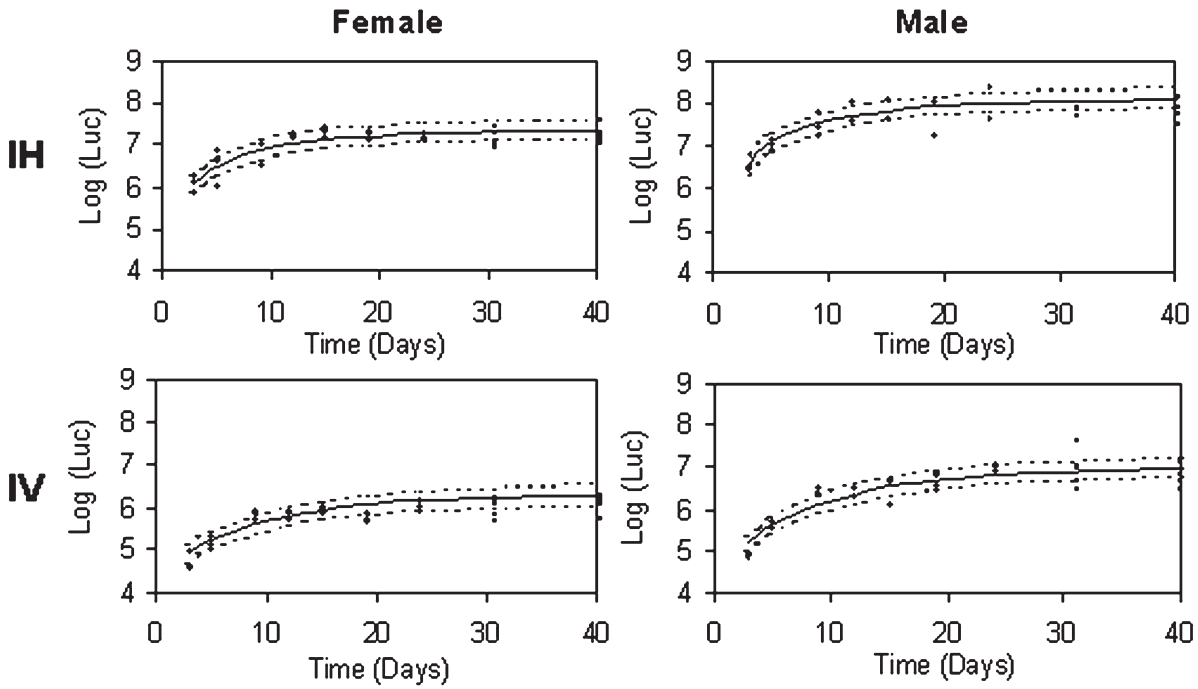

FIG. 2. Short-term *in vivo* CCD signal intensity in the liver of BALB/c mice injected with a high dose of AAV-Luc. Male and female BALB/c mice were injected with AAV serotype 2 expressing luciferase gene, at a dose of 2.5×10^{11} genome-containing particles. AAV-Luc was injected intravenously (IV; $n = 5$ males and 5 females) or intrahepatically (IH; $n = 5$ males and 5 females). Subsequent imaging was performed for luciferase expression, using D-luciferin injected intraperitoneally (150 mg/kg) and starting on day 3 and every week thereafter. Total bioluminescence was quantified and plotted over time. Each dot represents the decimal logarithm of bioluminescence for each animal at a time point. A solid line shows the mean value of each group; dashed lines indicate the confidence interval.

TABLE 2. L_{max} AND T_{50} VALUES FROM BALB/C MICE TRANSFER WITH 25×10^{11} VG OF AAV-LUC

	Female		*Male*	
	IV [M (SD)]	*IH[M (SD)]*	*IV[M (SD)]*	*IH[M (SD)]*
L_{max}	2.64 (0.036)	3.63 (0.062)	3.56 (0.034)	4.32 (0.183)
T_{50} (days)	5.83 (0.00020)	2.15 (0.00062)	5.83 (0.0020)	2.15 (0.00015)

Abbreviations: L_{max}, maximum increase of bioluminescence from baseline; M, Mean value; SD, standard deviation T_{50}, time necessary to reach the bioluminescence value $L_{max}/2$.

Because of the large range in luciferase expression, data were first subjected to logarithmic transformation before analysis. The logarithmic value of the luciferase expression (L) after AAV injection was modeled by assuming a saturable absorption process and a negligible elimination process in the time frame considered. The background level of bioluminescence (nonspecific light) was determined by acquiring the light of mice that had not been injected with luciferin, and was found to be equal to 4.

$$L = 4 + \frac{L_{max} \cdot t}{T_{50} + t}$$

where L_{max} represents the maximum increase in bioluminescence from baseline, T_{50} represents the time necessary to reach the bioluminescence value $L_{max}/2$, and t represents time.

Statistical analysis of the various estimated individual parameters was performed by two-way analysis of variance (ANOVA) followed by multiple comparison, using the least significant difference test (SPSS software; SPSS, Chicago, IL).

The AAV half-life in liver is determined by fitting the long-term follow-up data to an elimination process, assuming that the absorption process in this time frame is negligible:

$$L = L_{basal} \cdot e^{-k_e t}$$

where L_{basal} represents the bioluminescence value at steady state and k_e is the constant of the elimination process. The elimina-

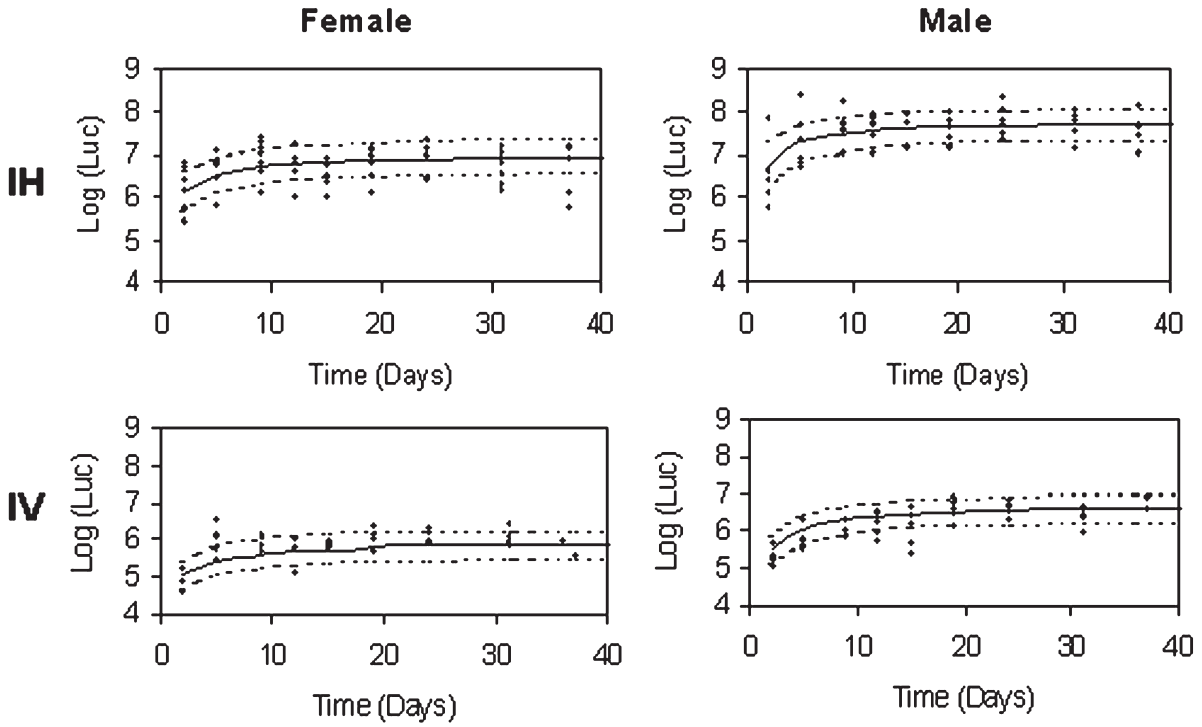

FIG. 3. Short-term *in vivo* CCD signal intensity in the liver of C57BL/6 mice injected with low-dose AAV-Luc. Male and female C57BL/6 mice were injected with AAV serotype 2 expressing luciferase gene, at a dose of 2.5×10^{10} genome-containing particles. AAV-Luc was injected intravenously (IV; $n = 5$ males and 5 females) or intrahepatically (IH; $n = 6$ males and 7 females). Subsequent imaging was performed for luciferase expression, using D-luciferin injected intraperitoneally (150 mg/kg) and starting on day 1 and every week thereafter. Total bioluminescence was quantified and plotted over time. Each dot represents the decimal logarithm of bioluminescence for each animal at a time point. A solid line shows the mean value of each group; dashed lines indicate the confidence interval.

TABLE 3. L_{max} AND T_{50} VALUES FROM C57BL/6 MICE TRANSFER WITH 2.5×10^{10} VG OF AAV-Luc

	Female		*Male*	
	IV [M (SD)]	*IH [M (SD)]*	*IV [M (SD)]*	*IH [M (SD)]*
L_{max}	2.05 (0.089)	2.93 (0.184)	2.64 (0.135)	3.88 (0.212)
T_{50} (days)	1.64 (0.121)	0.79 (0.359)	1.90 (0.095)	0.97 (0.445)

Abbreviations: L_{max}, maximum increase in bioluminescence from baseline; M, mean value; SD, standard deviation; T_{50}, time necessary to reach the bioluminescence value $L_{max}/2$.

tion half-life obtained from the estimated individual parameter k_e was compared by Kruskall–Wallis test.

RESULTS

In vivo *quantitative analysis of luciferase expression after AAV-Luc injection in liver*

Male and female BALB/c mice were injected with a single dose of AAV-Luc at 2.5×10^{10} VG using three different routes of administration: intravenous (IV; $n = 8$), intraportal (IP; $n = 3$), and direct injection into the liver (intrahepatic [IH]; $n = 8$). Whole body imaging was performed from day 1 postinjection up to day 92 postinjection and luciferase expression was quantified. Bioluminescence data were measured as photons/sec/cm^2/sr and analyzed in a pharmacokinetic model in which the "drug" is the transcriptionally active double-stranded DNA intermediate of the recombinant AAV. The effect of our drug is measured and represented as the logarithm of the bioluminescence value. This parameter is assumed to be proportional to the quantity of the drug. Quantitative data were fitted to a model of a saturable absorption process to perform a detailed statistical analysis (Fig. 1). In this model, we have defined L_{max} as the maximum increase in bioluminescence from baseline and T_{50} as the time necessary to reach the bioluminescence value $L_{max}/2$.

The results are summarized in Table 1. Analysis of L_{max} and T_{50} values from all the animals was performed with nonlinear mixed effect models. Model development was driven by the data; the value of the L_{max} parameter was affected by both gender and route of administration, whereas T_{50} was affected only by the route of administration. Statistical analysis of L_{max} and T_{50}, using the least significant difference (LSD) test with a significance level of 0.05, showed that on the basis of L_{max} four groups of animals showed significant differences: (1) IV female mice, showing the lowest value; (2) IP female mice and (3) IH female mice and IP and IV male mice, showing lower and higher intermediate values, respectively; and (4) IH male mice, showing the highest transgene expression values (Table 1).

The T_{50} value was affected by route, but was independent of gender. Therefore, three different subsets of animals could be distinguished: (1) IV mice, the subset requiring the longest time to reach this value; (2) IP mice, the intermediate subset; (3) IH mice, the third subset, expressing the transgene with the fastest kinetics. The most striking observation from the analysis of this parameter is the short period of time required to reach T_{50} after IH injection (Table 1).

Our data indicate that IH injection clearly improves AAV-mediated liver transduction in BALB/c mice, using a relatively low viral dose. To further confirm our observations we tested the effect of the route of administration, using a higher dose of virus and a different strain of mouse, C57BL/6. In these studies we analyzed IH and IV routes of administration.

Male and female BALB/c mice ($n = 5$) were injected IH and IV with a single dose of AAV-Luc at 2.5×10^{11} VG. Bioluminescence data were measured from day 3 postinjection up to day 40 postinjection and analyzed as previously described. As shown in Fig. 2 and Table 2, the differences in L_{max} shown by female mice were reduced, depending on the route of administration, when the dose of virus was increased; however, they were still significant (females: L_{max} IH = 3.63 versus L_{max} IV = 2.64; $p > 0.05$). Regarding T_{50}, as shown in Table 2, the values are identical to those obtained when using the low dose, indicating that the kinetics of transgene expression are independent of the dose of virus administered.

To analyze the effect of mouse strain on our results, male and female C57BL/6 mice ($n = 5$) were injected IH and IV with a single dose of AAV-Luc at 2.5×10^{10} VG. Bioluminescence data were measured from day 3 postinjection up to day 37 postinjection and analyzed as previously described. As shown in Fig. 3 and Table 3, as previously observed, the lowest expression level was achieved in female mice after IV injection. IH injection resulted in the highest levels of transgene expression in both male and female mice, with the luciferase expression levels of IH female mice becoming equivalent to those of IV male mice. Regarding the T_{50} parameter, the kinetics of transgene expression were faster after IH injection in both groups; interestingly, T_{50} values were smaller than in BALB/c mice after IV and IH injection, indicating faster expression kinetics (Table 3).

Comparison of transgene expression when steady state levels were reached

Luciferase transgene expression increased over time until steady state levels were reached approximately 4 weeks after rAAV administration in all the groups of female mice. Thereafter there was stable expression for more than 17 months (Fig. 4) (length of the study). L_{basal}, which is the bioluminescence value at steady state, and k_e, the constant of the elimination process, were calculated for every group (Table 4). The intensity of the signal slowly declined over time, and fitting the data to an exponential elimination model allowed us to determine a median elimination half-life of 31 years for the IV-injected animals, 55 years for the IP-injected animals

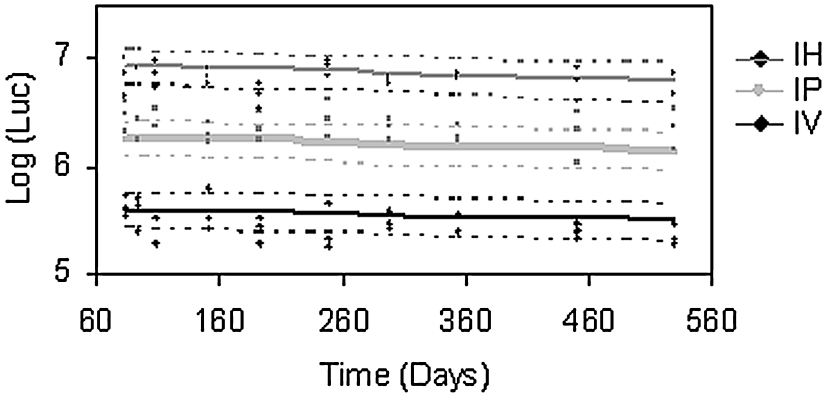

FIG. 4. Long-term *in vivo* CCD signal intensity in the liver of BALB/c mice injected with low-dose AAV-Luc. Female BALB/c mice ($n = 3$) were injected with AAV serotype 2 expressing luciferase gene at a dose of 2.5×10^{10} genome-containing particles. AAV-Luc was injected IP, IV, or IH. Each dot represents the decimal logarithm of the bioluminescence value for each animal at each time point. A solid line shows the mean value of each group; dashed lines indicate the confidence interval.

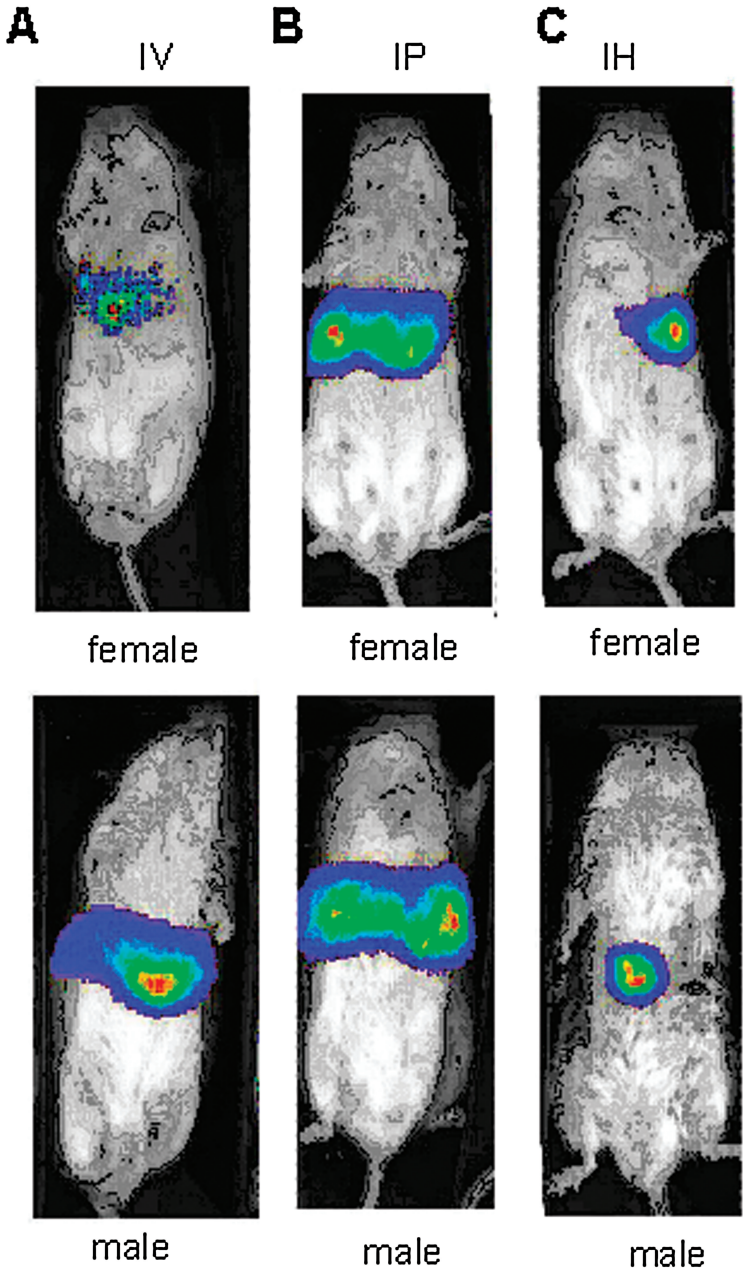

FIG. 5. Bioluminescence imaging of AAV-Luc gene expression in living mice, using a cooled CCD camera. Eight-week-old male and female BALB/c mice were injected with AAV serotype 2 expressing the luciferase gene, at a dose of 2.5×10^{10} genome-containing particles and using different routes. The mice were repeatedly scanned with the CCD camera as described in Fig. 2. Optical CCD images for luciferase expression 96 days postinjection, once a steady state level of luciferase expression was reached, are shown. (**A**) Male and female mice injected intravenously (IV); (**B**) male and female mice injected intraportally (IP); (**C**) male and female mice injected intrahepatically (IH).

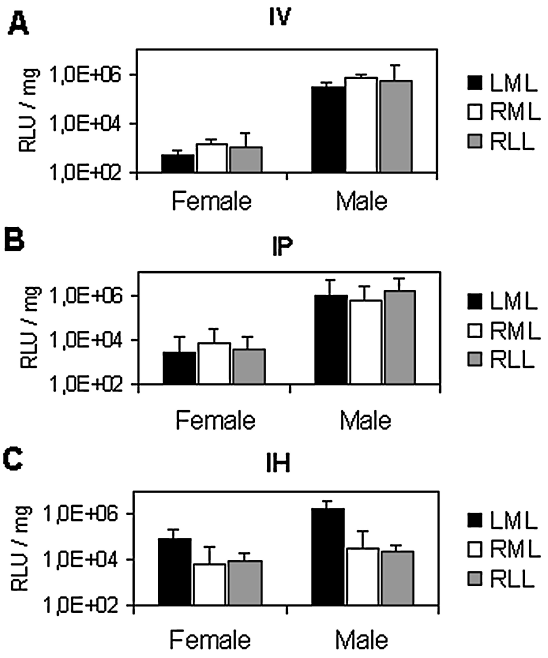

FIG. 6. *Ex vivo* analysis of luciferase expression in liver lobes. Female and male BALB/c mice were injected with 2.5×10^{10} genome-containing particles of AAV-Luc by three different routes (IV, IP, and IH). Ninety-two days later, mice were killed and three of the hepatic lobes were removed: the left medial lobe (LML) (injected lobe), the right medial lobe (RML), and the right lateral lobe (RLL). Luciferase expression was tested in the homogenized tissue, using a luminometer. The *y* axis indicates the median relative light units (RLU) per milligram of protein of each group. (**A**) IV injected animals; (**B**) IP injected animals; (**C**) IH injected animals. After IH injection the injected lobe showed the highest luciferase expression value ($p < 0.05$; ANOVA); after IV and IP injection luciferase activity was uniformly distributed throughout the main hepatic lobes.

and 35 years for the IH-injected animals (Table 4), which means life-long expression of the transgene in the liver of all animals after AAV injection. Differences in the half-life value among the groups were not statistically significant, indicating that the elimination process does not depend on the route of administration.

Biodistribution of luciferase expression

As shown in Fig. 5, by the time luciferase expression reached steady state levels in the liver, no luciferase expression could be detected in other organs independently of the route of administration or gender. Furthermore, expression of luciferase was evenly distributed throughout the entire liver after IV and IP administration of AAV-Luc (Fig. 5A and B), whereas IH administration of the virus resulted in higher expression of luciferase at the site of injection (Fig. 5C).

To confirm and supplement *in vivo* imaging data, animals from the first study (BALB/c mice injected with 2.5×10^{10} VG of AAV-Luc) were killed 3 months after AAV injection and whole organ preparations were analyzed *ex vivo* for luciferase activity, using a luminometer. To perform the analysis of luciferase expression, the three major lobes of the liver were excised: left medial lobe (LML), right medial lobe (RML), and right lateral lobe (RLL). We also tested luciferase expression in other organs, such as testicles or ovaries, spleen, stomach, lungs, heart, kidney, and injected muscle. As shown in Fig. 6, *ex vivo* analysis confirmed the findings based on bioluminescence image analysis. Luciferase expression after IH injection was confined mainly to the injected left medial lobe. Expression in the other lobes was more than 100-fold lower. However, luciferase expression in the liver of IV- and IP-injected mice was evenly distributed. Analysis of luciferase expression in the rest of the organs tested revealed no luciferase expression after IH injection. Low levels of luciferase expression were detected in the spleen and heart of male mice given IV injections (278.60 and 1585.73 RLU/mg of tissue protein, respectively) and in the spleen and kidney of male mice given IP injections (647.71 and 6203.65 RLU/mg of tissue protein, respectively).

DISCUSSION

We constructed an rAAV vector that expresses luciferase under the transcriptional control of a constitutive promoter to evaluate *in vivo* the effect of the route of administration on AAV-mediated liver transduction. A detailed and quantitative long-term *in vivo* follow-up of the biodistribution of transgene expression after the administration of rAAV was performed. The bioluminescence data were analyzed using a pharmacokinetic model in which the "drug" was the transcriptionally active double-stranded DNA intermediate of the recombinant AAV and the "effect" of the drug was measured and represented as the logarithm of the bioluminescence value. This parameter was assumed to be proportional to the quantity of the drug. Quantitative data were fitted to a model of a saturable absorption process to perform a detailed statistical analysis. Our results show that luciferase expression levels in the liver clearly depend on the route of AAV administration and the gender of the animal, whereas the kinetics of AAV-mediated transgene expression were affected by the route of administration and, in-

TABLE 4. L_{basal} AND k_e VALUES FROM BALB/c FEMALE MICE TRANSFER WITH 2.5×10^{10} VG OF AAV-Luc

	IV [M (SD)]	*IP [M (SD)]*	*IH [M (SD)]*
L_{basal}	5.575 (0.089)	6.377 (0.105)	6.904 (0.046)
k_e (years^{-1})	0.0230 (0.020)	0.0081 (0.0151)	0.0195 (0.070)

Abbreviations: L_{basal}, bioluminescence value of steady state; M, mean value; SD, standard deviation; k_e, constant of the elimination process.

terestingly, by the strain of mouse and was independent of the sex. All animals demonstrated life-long luciferase expression with no evidence of vector silencing or immune response against the transgene, even though we were using a nonmammalian protein.

As previously described (Davidoff *et al*., 2003; Berraondo *et al*., 2005), differences in the magnitude of transgene expression were found in the liver after IV administration of rAAV-2, depending on the gender of the animal (Fig. 1). However, we also found that these differences could be minimized by modifying the route of administration. Intraportal injection reduced the difference from 100-fold to less than 10-fold and IH injection further reduced the difference to less than 3-fold. Those results, obtained with a relatively low dose of virus, were reproduced with a 10-fold higher amount (Fig. 2) and were independent of the strain of mouse (Fig. 3). Interestingly, IV and IP injection procedures in male mice resulted in equivalent transgene expression, whereas IH injection resulted in the highest level of transgene expression. Thus IH injection clearly improves liver transduction independently of gender, the dose of virus, or the strain of mouse.

The mechanism underlying the lower efficiency of AAV-mediated liver transduction observed in female mice is currently unknown. Davidoff *et al*. (2003) suggested that the differences were due to an androgen-dependent pathway, associated with the presence of male-specific cellular factors that lead to higher level of rAAV genomes in male than in female mice. The data obtained from our study showed that the differences in gene expression depending on gender were minimized when the virus was administered by liver-directed injection. These results suggest that increasing the local concentration of rAAV in the liver might overcome the lack of certain androgen-related factor(s) or, alternatively, that IH injection induces the expression of those factors, necessary for the establishment of AAV-mediated transgene expression in females. However, we cannot rule out the possibility that liver-directed injections might bypass the inhibitory effect of factors present in the blood of animals.

Furthermore, the analysis of transgene expression in mice given IH injections showed a faster onset of luciferase expression in comparison with mice given IV and IP injections. The rate-limiting steps of AAV transduction efficacy are the relatively slow nuclear import of AAV genomes, the rate of virus uncoating, and the conversion of ssDNA to transcriptionally competent double-stranded templates (Alexander *et al*., 1994; Ferrari *et al*., 1996; Bartlett *et al*., 2000; Chen *et al*., 2001; Thomas *et al*., 2004). In the liver, it has been suggested that ds-DNA conversion occurs through annealing of complementary plus and minus single-stranded molecules, not by the *de novo* synthesis of the second strand; if this is the case the rate of uncoating is a key determinant for the ability of complementary molecules to convert to biologically active molecular forms (Nakai *et al*., 2000). IH injection might be associated with faster entrance of AAV genomes into the nucleus or with a faster uncoating of vector genomes, which would allow, as in the case of other pseudotyped vectors (Thomas *et al*., 2004), a more efficient formation of AAV double-stranded templates. However, there is still a certain degree of controversy regarding the mechanism of the formation of transcriptionally active forms and other explanation may be plausible as well (Qing *et al*., 1998; Miao *et al*., 2000; Nakai *et al*., 2000).

The explanation we favor, on the basis of our observations, is that intrahepatic injection first reduces the contact of AAV vectors with inhibitory molecules present in the blood and concentrates the virus in the liver. Once in the liver, the virus undergoes faster uncoating (by a mechanism that requires further studies to be elucidated), resulting in faster formation of transcriptionally active forms.

The *in vivo* and *ex vivo* analysis of luciferase expression after intravenous or intraportal AAV administration showed a wide distribution throughout the liver and a low level of expression in some organs in male but not in female mice (data not shown). This might indicate that the gender dependency detected in the liver also occurs in other organs (Dodge *et al*., 2005). After intrahepatic injection, luciferase expression is restricted mostly to the injected hepatic lobe, with low levels of expression in the other hepatic lobes and nondetectable levels of expression in the rest of the organs analyzed.

In summary, AAV intrahepatic injection offers several advantages over other routes of administration: faster transgene expression, maximum protein expression, and expression confined to the injected hepatic lobe. Finally, direct intrahepatic injection of the virus by ultrasonographic guidance could be easily performed in humans and in large animals. All these factors point to the potential of this route of administration for future clinical applications.

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