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Modified Infusion Procedures Affect Recombinant Adeno-Associated Virus Vector Type 2 Transduction in the Liver

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ABSTRACT

Recombinant adeno-associated virus (rAAV) vectors have therapeutic potential for the treatment of several types of liver diseases including hepato-deficiency disorders. Most of the preclinical and clinical applications involve the use of adeno-associated vector serotype 2 (AAV-2). However, when this vector is delivered at high doses into the portal vein or hepatic artery, a relatively small number of hepatocytes are stably transduced. We elected to determine if the route of vector administration and altering the vascular delivery route within the liver influenced the relative level of transduction. First, we delivered an AAV vector expressing the human factor IX gene from a liver-specific promoter into the hepatic artery, portal vein, or general circulation of rats. Transgene expression was equal with hepatic artery and portal vein infusion, which was higher than vector administered via peripheral venous infusion. Next, we determined how localized perfusion or changing the vector dwell time affected AAV transduction *in vivo*. To do this, we infused an AAV vector lacking a functional expression and quantified transduction by quantifying the number of double-stranded vector DNA genomes. By increasing vector dwell time in the liver to 5 min, vector transduction was enhanced approximately 4- to 5-fold. To establish if gene transduction could be restricted to a specific anatomic location in the liver, we delivered vector into specific liver lobes by clamping the venous inflow to the middle and left liver lobes (noninfused lobes) and infusing vector into the right two liver lobes through the hepatic artery followed by vector circulation between the two right lobes and general circulation for 5 min. With this selective infusion, 40 to 120 times higher vector genome was observed in the perfused lobes than the nonperfused lobes. All the procedures described in this study were performed without detectable liver injury or toxicity. In all, the present study clearly demonstrated that hepatic arterial infusion of rAAV is effective for liver-directed gene therapy and that other parameters related to blood flow can be adjusted to further optimize gene transfer.

OVERVIEW SUMMARY

Hepatic gene transfer for adeno-associated vector serotype 2 (AAV-2) vectors show promise for gene therapy trials. We established that blood flow and the vascular route of vector administration can influence transduction levels *in vivo*.

INTRODUCTION

AMONG SEVERAL VIRAL VECTOR SYSTEMS developed to date, recombinant adeno-associated virus (rAAV) is a promising vehicle for delivery of transgenes to various tissues, because of its success in animal models of human disease and relative good safety profile (Snyder *et al*., 1999; Kay *et al*., 2000, 2001; Arruda *et al*., 2001; Davidoff *et al*., 2002; Manno *et al*., 2003).

There are many different AAV serotypes and to date a number have been studied to varying degrees for gene therapy (Gao *et al*., 2002, 2004; Grimm *et al*., 2003). Among those, adeno-associated vector serotype 2 (AAV-2) is the most extensively studied and used in preclinical and clinical studies. Recent experiments have shown that rAAV-2 at doses of 5×10^{12} vg/kg delivery to the liver succeeded in curing or phenotypic alterations in diseases models of hemophilia (Snyder *et al*., 1997, 1999; Kay *et al*., 2000; Wang *et al*., 2000; Mount *et al*., 2002; Nathwani *et al*., 2002), glycogen storage disease (Beaty *et al*.,

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2002), mucopolysaccharidosis (Elliger *et al*., 2002) and cancer (Davidoff *et al*., 2002). However, one interesting problem that arose in previous experiments is that even at higher rAAV doses, the liver transduction efficiency is low (the proportion of hepatocytes stably transduced is limited to 5–10%) (Miao *et al*., 1998, 2000; Chen *et al*., 2001; Nakai *et al*., 2002). In this context, we wanted to determine how important delivery methods are for achieving optimal transduction *in vivo*.

In most of the previously reported experiments, vectors have been delivered to the liver through the portal circulation. This is because with AAV-2 vectors, peripheral intravenous infusion resulted in lower levels of gene transfer *in vivo* (Snyder *et al*., 1997; Grimm *et al*., 2003). However, for clinical application, portal vein infusion is a complex procedure involving a percutaneous transhepatic approach, whereas for hepatic artery infusion, the method is simple and involves catheter insertion into the femoral artery and then into the hepatic artery guided by routine fluorographic procedures. The risk of delivery must be weighed in comparison to the relative efficiency of gene transfer. Thus, in the present study, a technique to insert a microcatheter into the proper hepatic artery of rats that allows hepatic arterial delivery in small animals was developed. We first delivered rAAV vector to the liver through three different routes—intravenous injection, portal vein infusion or hepatic artery infusion—to determine the optimal delivery route in terms of transgene expression. We then modified the hepatic arterial infusion procedure to increase the dwell time of the vector to determine if this affected the level of transduction *in vivo*. Finally, we altered the vascular route of vector administration in the liver to attempt to determine if we could achieve selective liver lobe delivery.

MATERIALS AND METHODS

Animals

Male nude rats, weighing 140–160 g and inbred male adult Lewis rats (18–20 weeks old), weighing 440–480 g, or young Lewis rats (6–7 weeks old), weighing 200–240 g, were purchased from Harlan (Indianapolis, IN). All animal protocols were performed according to the Stanford University and National Institutes of Health (NIH) guidelines. Rats were housed with access to water and standard laboratory chow *ad libitum*.

Vector preparation

Description for the AAV-ApoE/hAAT-hFIX vector has been previously described (Grimm *et al*., 2003).

The AAV-null vector was purified by heparin column chromatography in the vector core facility at Avigen Inc (Alameda, CA). This vector contains a nonfunctional β-galactosidase–neomycin phosphotransferase expression cassette (Arruda *et al*., 2001). The physical particle titers were determined by a quantitative dot-blot assay as described previously (Kessler *et al*., 1996).

Vector infusion procedure and experimental groups

Under general anesthesia with isoflurane (Abbott Laboratories, North Chicago, IL), the following microscopic surgical procedures were carried out aseptically using a Nikon surgical microscope. After laparotomy with a midline abdominal incision, the common hepatic artery (CHA), proper hepatic artery (PHA), and gastroduodenal artery (GDA) were isolated in the following three hepatic artery infusion procedures.

Hepatic artery infusion procedure (HA group). As a general hepatic artery infusion procedure (HA group), we temporarily clamped the CHA with a microsurgical clip (Weck, Research Triangle Park, NC) and then inserted thin PE-10 catheter into the PHA through a cut-down of the GDA. AAV vector at a dose of 5×10^{12} vg/kg was infused into the hepatic artery through the catheter over a period of approximately 1 min. After the vector infusion, the PE-10 catheter was removed and the cut-down of the GDA was ligated and then the clip on the CHA was removed. Before closing the abdominal incision, we confirmed under the microscope that the arterial blood flow on the PHA was restored.

Hepatic artery infusion with outflow block procedure (OB group). In order to assess whether the increase in the dwell time of the infused vector in the liver will affect the transduction efficiency, we temporarily clamped the venous outflow of the liver (OB group). In detail, the PE-10 catheter was inserted to the PHA through the GDA as described in the HA group. Suprahepatic vena cava (SHVC), infrahepatic vena cava (IHVC, at the proximal side of the renal veins) and portal vein (PV) were isolated. Just before the vector infusion, we clamped the PV and the SHVC together with hepatic veins. We also clamped the IHVC in order to avoid the possible venous outflow through the short hepatic veins. AAV vector at a dose of 5×10^{12} vg/kg was infused into the hepatic artery over a period of 1 min. With this procedure, the liver does not have venous inflow (except for the AAV vector solution) nor venous outflow. Five minutes after the infusion, we removed the clamps on the SHVC and IHVC and then the PV. PE-10 catheter removal and abdominal closure was as described in the HA group.

Hepatic artery infusion with selective liver lobes infusion procedure (SE group). In order to obtain AAV-mediated liver transduction in specific liver lobes, we temporarily restricted the blood

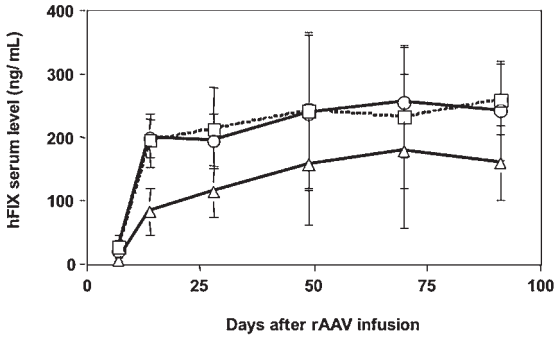

FIG. 1. Human clotting factor IX expression level in the serum of rats after administration of 5×10^{12} vg/kg of AAV-ApoE/hAAT-hFIX via hepatic artery (circle), portal vein (square), or general circulation through tail vein (triangle). Serum samples were collected over time and assayed for hFIX (n = 3–4 per group).

circulation into those specific lobes (SE group). In detail, the PE-10 catheter was inserted into the PHA through the GDA as described in the HA group. Left portal branch (LPV) and left hepatic artery (LHA) were isolated. Just before the vector infusion, we clamped the LPV together with the LHA using the microsurgical clip. AAV vector at a dose of 5×10^{12} vg/kg was infused into the hepatic artery over a period of 1 min. Five minutes after the infusion, we removed the clamps on the LPV and LHA. With this procedure, the two left liver lobes (lateral plus median lobes) did not have blood access for 5 min and the AAV vector contact to the liver was limited to the right liver lobes. PE-10 catheter removal and abdominal closure was as described in the HA group.

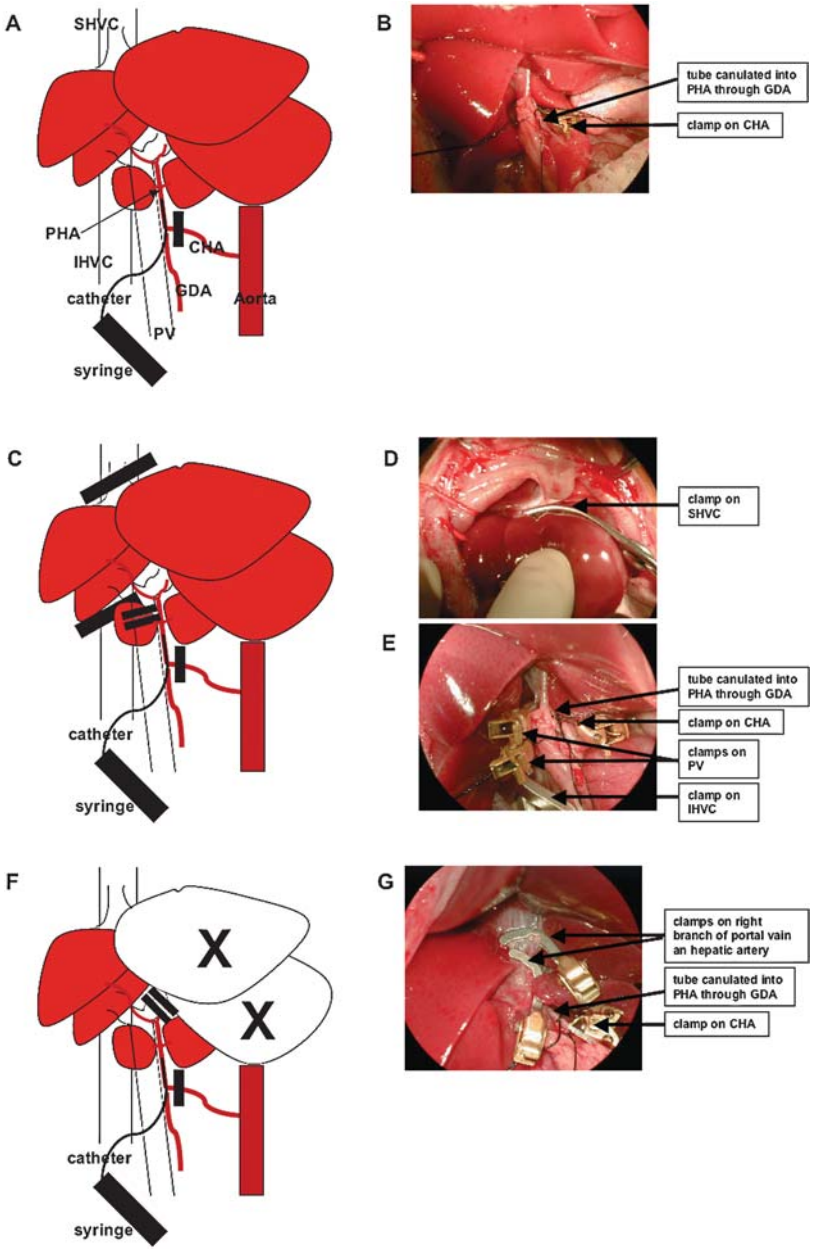

FIG. 2. Schematic illustration (**A**, **C**, **F**) and operation field (**B**, **D**, **E**, and **G**) of the adeno-associated virus (AAV) vector hepatic artery infusion procedures. **A** and **B**: Hepatic artery infusion procedure (HA group). A PE-10 catheter was inserted into the PHA through a cut-down of the GDA. **C**–**E**: Hepatic artery infusion with outflow block procedure (OB group). SHVC, IHVC, and PV were temporarily clamped during the vector infusion and 5 min after the vector infusion. **F** and **G**: Hepatic artery infusion with selective liver lobes infusion (SE group). Left portal and hepatic artery branches were temporarily clamped during the vector infusion and 5 min after the vector infusion. Liver lobes that have blood access are colored in red and those without blood access are indicated with an X. Note the color of the liver lobes without blood access turned into purple compared with lobes with blood access. SHVC, suprahepatic vena cava; IHVC, infrahepatic vena cava; PHA, proper hepatic artery; CHA, common hepatic artery; PV, portal vein; GDA, gastroduodenal artery.

Portal vein infusion procedure (PV group). Portal vein AAV infusion was performed as described previously (Park *et al*., 2000a). After laparotomy, AAV vector at a dose of 5×10^{12} vg/kg was infused into the portal for over a period of 1 min.

Infusion into the general circulation through the tail vein (TV group). AAV vector was infused into the general circulation through the tail vein by inserting the 27G syringe needle into the vein. Vector at a dose of 5×10^{12} vg/kg was infused for over a period of 1 min.

Assay for hFIX level in the serum. Human FIX concentrations in rat serum were determined by an enzyme-linked immunosorbent assay (ELISA) using an antibody specific for hFIX as described previously (Park *et al*., 2000b). The detection limit of this assay is 2 ng/ml.

Assay for serum alanine aminotransferase (ALT)

In order to observe if there was a procedure-related liver injury, rat serum was collected at day 2, day 7, and day 21 after the vector infusion. The serums were stored at 4°C until the time of the assay. Rat serum ALT concentration was determined by colorimetric diagnostic kit (Sigma, St. Louis, MO) as described previously (Park *et al*., 2000a).

Organ collection and DNA analysis

At 7 weeks after vector infusion, the rats were euthanized by overdose anesthesia with isoflurane. For the HA and PV groups, the whole liver were harvested; for the OB and SE groups the two right liver lobes and the left liver lobes (lateral plus median lobes) were harvested separately for the DNA analysis. Extraction of total genomic DNA from rat livers, Southern blot analysis, and densitometric analysis of the blots were performed as previously described (Nakai *et al*., 1999), to determine vector genome number per diploid genomic equivalent (vg/dge). The vector genome copy number standards were prepared by adding an equivalent number of corresponding plasmid molecules to 20 μg of total DNA extracted from naïve mouse liver.

Statistical analysis

The significance of differences between groups was tested by a one-way analysis of variance (ANOVA) with the use of StatView 5.0 software (SAS Institute Inc., Cary, NC).

RESULTS

Transgene expression by three different routes of rAAV delivery in rats

Nude rats received AAV-ApoE/hAAT-hFIX at a dose of 5×10^{12} vg/kg via three different routes, namely, hepatic artery, portal vein, and tail vein (HA, PV and TV groups, respectively) (Fig. 1). One week after the infusion, rats in the HA and PV groups, but not the TV group, had detectable amounts of hFIX in the serum (15–25 ng/ml). The serum hFIX level gradually increased thereafter, the HA and PV group reaching a steady-state level of 200–300 ng/ml of hFIX (4–6% of normal levels in human serum). At all the time points, the serum hFIX levels were similar between the HA and PV group but approximately two times higher than the TV group. This confirmed that HA or PV delivery route is more efficient at rAAV-mediated transduction than the infusion into the general circulation.

Safety of varied vector perfusion strategies

For hepatic gene transfer, vectors have been infused through the hepatic artery or portal vein. Because we confirmed that HA infusion is equally effective for rAAV-mediated gene transduction, this study was undertaken to establish alternative AAV delivery methods as a means for enhancing transduction to the liver *in vivo*. To do this, we used immunocompetent Lewis rats, 18–20 weeks of age, weighing 440–480 g and infused AAV-null vector at a dose of 5×10^{12} vg/kg through the hepatic artery under different conditions. The HA, OB, and SE groups are as described in the Materials and Methods section (Fig. 2A–2G). The nontransgene expressing AAV-null vector contains a nonfunctional β-geo (β-gal and neo fusing) gene and was used to avoid any loss of rAAV-vector transduced cells by cytotoxic T lymphocyte (CTL)-mediated immune response against transgene-expressing cells. With the infusion methods established in the present study, there was no procedure-related or vector infusion-related mortality observed in any of the groups.

To monitor the success of the infusion procedure, we observed the liver color as a parameter of blood perfusion under a surgical microscope. There were no vector-infusion related color changes in the HA and PV groups (Fig. 2B). In the OB group, the blood access of the liver was temporarily shut down

Table 1. Alanine Aminotransferase Activity (I.U.) in Rats Receiving AAV Infusion Through Hepatic Artery or Portal Vein

Vector infusion procedure	*No. of rats*	*Alanine aminotransferase activity (I.U.)*		
		Day 2	*Day 7*	*Day 21*
HA group	3	39.1 ± 6.8	36.0 ± 6.4	40.7 ± 5.1
OB group	4	38.4 ± 6.2	41.3 ± 6.5	37.3 ± 4.4
SE group	4	33.9 ± 7.4	36.7 ± 5.1	33.5 ± 3.7
PV group	3	38.3 ± 7.4	35.5 ± 3.9	38.0 ± 4.5
Sham group	3	38.3 ± 8.1	40.5 ± 7.4	36.8 ± 6.9

HA, hepatic artery infusion procedure; OB, hepatic artery infusion with outflow block procedure; SE, hepatic artery infusion with selective liver lobes infusion procedure; PV, portal vein infusion procedure. Values are mean ± S.D.

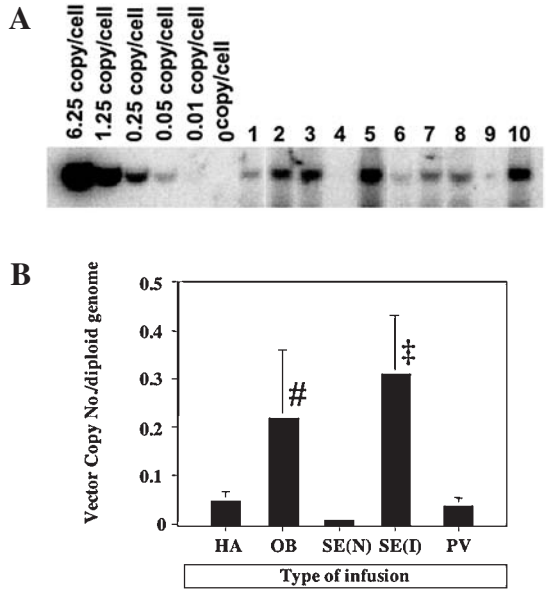

FIG. 3. **A:** Southern blot analysis to determine recombinant adeno-associated virus (rAAV) vector copy number in the livers of adult rats (440–480 g in weight) injected with 5×10^{12} vg/kg of AAV-null vector into the liver through the hepatic artery with different modification. The rats were sacrificed 7 weeks postinjection, and whole liver (HA group) or right and left liver lobes (OB and SE group) were analyzed. Twenty micrograms of total DNA extracted from each sample was digested with *Bgl*I, electrophoresed, and hybridized with probe. Vector copy number standards were 20 μg of naïve rat liver DNA spiked with an equivalent number of the vector plasmid molecules and described as copies per cell (the number of double-stranded rAAV genomes per diploid genomic equivalent). Lanes 1 and 6, HA group. Lanes 2 and 7, left liver lobes of OB group. Lanes 3 and 8, right liver lobes of OB group. Lanes 4 and 9, left liver lobes (non-perfused liver lobes) of SE group. Lanes 5 and 10, right liver lobes (perfused liver lobes of SE group). **B**: Vector copy number in the livers of adult rats in the HA, OB, SE and PV groups. Values are mean ± standard deviation (SD) $\#p <$ 0.01 (OB vs. HA or PV groups). $\ddagger p < 0.01$ (SE(I) vs. SE(N)). HA, hepatic artery infusion procedure; OB, hepatic artery infusion with outflow block procedure; SE, hepatic artery infusion with selective liver lobes infusion procedure; SE(N), non-vector–infused left liver lobes; SE(I), vector-infused right liver lobes; PV, portal vein infusion procedure. (n = 4–5 per group).

and the nonperfused liver became dark red, while the infused liver lobes became slightly white in color during the period of infusion (Fig. 2D). The liver color returned to normal in this group after the clamps were removed and blood flow restored. In the SE group, the color of the left and median liver lobes was temporarily purple because these lobes did not have blood flow during the procedure (Fig. 2G). Immediately after the clamps were removed, the liver color returned to normal. To determine if any vector-infusion or procedure related liver injury occurred, we measured serum ALT, a sensitive marker for liver injury, 2, 7, and 21 days after the vector infusion. As shown in Table 1, no significant elevation of the ALT values were observed in any of the groups, suggesting that these procedures could be performed without causing significant hepatocellular injury.

Quantitative evaluation of AAV-mediated liver gene transfer

To provide a quantitative evaluation of gene transfer to the liver lobes, we determined AAV copy number by Southern blotting using liver samples taken 7 weeks after the vector infusion. As shown in Figure 3B, similar stable double-stranded vector genome copy numbers were obtained in the HA and PV groups (0.05 ± 0.02 and 0.04 ± 0.01 vector copy per diploid genome, respectively). As shown in Figures 3A and 3B, when we increased the dwell time of the infused AAV vector in the liver using outflow block procedure (OB group), there was a 5-fold increase (0.22 ± 0.14) in the number of vector genomes compared to simple hepatic artery infusion (HA group). We then determined if we could deliver AAV to the selective liver lobes. To do this, we restricted blood access to the right two liver lobes during and 5 min after the vector infusion (SE group).

With this procedure, we found a significantly higher level of transduction in the perfused lobes (0.31 ± 0.12 vector copy per diploid genome) compared to the whole liver arterial (HA) or portal (PV) administration. Furthermore, we minimized vector gene transfer to the nonperfused liver lobes to 0.01 ± 0.01 vg/dge.

Age-related differences in the AAV transduction to the livers

We determined if the age-difference in the liver transduction could be observed by rAAV hepatic arterial or portal infusion. Lewis rats at age 6–7 weeks, weighing 200–240 g received AAV-null vectors at a dose of 5×10^{12} vg/kg through the hepatic artery with HA, OB, SE, and PV procedures. As shown in Figures 3 and 4, the rAAV copy number in the liver of young rats was approximately 25–35% of the copy number detected in the adult rats. We believe this was because of the continued growth of the rat liver resulting in the dilution of episomal AAV genomes at this age (Nakai *et al*., 2001; Gao *et al*., 2004). We then studied whether higher gene transfer in the OB group and

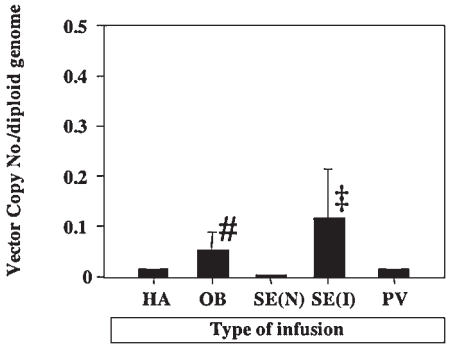

FIG. 4. Vector copy number determined by Southern blot analysis in the livers of young rats (220–260 g in weight) of the HA, OB, SE, and PV groups. Values are mean ± standard deviation (SD). $\#p < 0.05$ (OB vs. HA or PV groups). $\ddagger p <$ 0.01 (SE(I) vs SE(N)). HA, hepatic artery infusion procedure; OB, hepatic artery infusion with outflow block procedure; SE, hepatic artery infusion with selective liver lobes infusion procedure; SE(N); non-vector–infused left liver lobes; SE(I), vector-infused right liver lobes; PV, portal vein infusion procedure. (n = 3–4 per group).

selective gene transfer in the SE group could also be obtained in the young rats. As with the adult rats (Fig. 3B), the young OB group showed a *5* times (0.05 vs. 0.01 vector genomes per cell) higher level of gene transfer than the HA or PV group ($p < 0.01$) in the young rat liver. In the SE group, the perfused lobes showed a relatively high level of gene transfer (0.12 ± 0.11), while almost no vector genome could be detected in the nonperfused liver lobes.

DISCUSSION

rAAV-2 vectors are more efficient at gene transfer when delivered into the hepatic versus systemic circulation (Snyder *et al*., 1997). Because we used a hepatocyte-specific promoter, all of the transgene expression was obtained from hepatocytes and not from cells that may have been transduced in other tissues. This study established that the vector was equally efficient at gene transduction when delivered via the hepatic artery or portal vein. This is important because it demonstrates that subtle differences in the blood flow in the liver from these two vessels does not influence the transduction efficiency with AAV-2 vectors. Furthermore, it substantiates that the less invasive and less risky hepatic artery infusion is equally viable from a clinical trial standpoint. Although Southern blot analyses have shown that liver-directed rAAV-2 delivery could be obtained by portal vein as well as tail vein infusion, the present result suggests that a high concentration of rAAV contact in the liver is an important factor for AAV-mediated liver gene therapy. The present study also showed that rAAV delivery through the hepatic artery gave similar levels of transgene expression compared to portal vein delivery. In the clinical setting, portal infusion requires catheter insertion into the portal vein by puncturing the liver under the ultrasound or computed tomography guidance. This procedure has a risk of intra-abdominal bleeding, because the liver is a blood flow-rich organ. Because of this unwanted possibility, portal infusion is hard to apply to patients who have bleeding disorder such as hemophilia. In contrast, hepatic arterial infusion has been safely performed as a routine procedure by inserting a catheter into the hepatic artery via the femoral artery. Given this information, it is reasonable to conclude that hepatic arterial infusion is the method of choice for AAV-2 mediated liver gene therapy. We also confirmed that rAAV transduction was enhanced by increasing the vector dwell time and selective infusion could be achieved in young rat livers.

In our study there was no difference in liver transduction between portal vein and hepatic artery infused animals. However, in a non-human primate study, portal vein infused animals had more vector than hepatic artery treated animals (Nathwani *et al*., 2002). Complicating issues included the fact that the hepatic artery treated animals had higher pretreatment anti-AAV antibody titers prior to vector infusion and there may have been some complications with the delivery in one of the artery-infused animals. Because blood flow into the microenvironment of the liver is similar between the portal vein and hepatic artery, we anticipate that under equal conditions transduction should be the same. Because AAV-2 is taken up by heparin binding molecules, vector infused into the tail vein is likely taken up by numerous tissues before reaching the liver parenchyma resulting in lower hepatic transduction.

We also showed that there was no observable vector infusion-associated liver toxicity or liver damage in both the arterial and portal delivery groups. The novel technique described in this study to insert an ultrathin catheter into the proper hepatic artery in rats allowed consistent infusion without any bleeding. Furthermore, we can easily confirm that hepatic arterial blood flow reconstitution under the surgical microscope is one of the important factors to prevent hepatic injury after the procedure. In addition to the provision of a vector by one-shot infusion, we could deliver vector or agents continuously into the hepatic artery when the catheter is connected to the continuous infusion pump system (Patijn *et al*., 1998). A number of rat studies including, pharmacologic, toxicologic, and antiliver cancer therapy investigations could be performed with this system.

In the initial experiment, HA and PV infusion induced higher levels of expression than TV infusion, which strongly suggested that first pass metabolism in the liver was an important factor. As a result, we increased the dwell time in the liver as a means for enhancing transduction *in vivo*. By increasing the dwell time to 5 minutes, we enhanced transduction by about 5-fold. However, it is not clear if longer dwell times would further increase gene transduction. It is possible to increase the dwell time to 30 min without liver injury (Horikawa *et al*., 1994) but further studies are required to determine if this has any advantages. In the clinic, this outflow block approach could be performed at the time of abdominal surgery by totally isolating the liver from the blood circulation system. Liver isolation from the blood circulation, could also be performed using a recently developed fluorographic technique (Ku *et al*., 1998). These two methods have advantages that could restrict the vector contact to the liver, and we could discard the remaining vector from the body by filtering the outflow from the vena cava (Horikawa *et al*., 1994).

To date, studies have noted that rAAV effectively deliver genes to liver cancer cells *in vitro* (Peng *et al*., 2000; Su *et al*., 2000). However, the gene transduction efficiency to the liver tumors *in vivo* is quite low (Peng *et al*., 2000) so that additional strategies (e.g., radiotherapy) are required to enhance the transduction to the tumor or tumor harboring liver lobe. By restricting the blood access to certain liver lobes for 5 min, we obtained higher gene transduction in the perfused liver lobes compared to the regular arterial or portal whole liver rAAV delivery. Furthermore, we could minimize the transduction efficiency to a barely detectable level in the nonperfused liver lobes (40 to 120 interlobe difference in the rAAV genome). These findings suggest that vector binding and uptake occurs during the 5-min perfusion period and that most of the vector is taken up during first-pass metabolism. The selective infusion procedure will allow higher anticancer gene transduction to the target liver lobe(s) without causing disruption of the normal intact liver lobes. Although this selective infusion could be surgically or fluorographically performed in the clinic with currently developed techniques as described above, further studies are required to optimize physical parameters for optimizing selective vector transduction.

In summary, our present study have established new procedures to achieve dramatic enhancements in transduction efficiency in the liver *in vivo* by infusing rAAV through the hepatic artery. These procedures could be applicable to the clinical

setting. Such an intervention could be advantageous for the use or rAAV to treat a wide range of liver diseases.

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