Gene Ther Mol Biol Vol 9, 169-180, 2005

Mouse and pig nonviral liver gene therapy: success and trials

Research Article

Maria Jose Herrero, Francisco Dasi, Inmaculada Noguera, Maria Sanchez, Ines Moret, Isaias Sanmartin, Salvador F. Aliño*

Departamento de Farmacología, Facultad de Medicina, Universidad de Valencia; Valencia (Spain)

***Correspondence**: Salvador F. Aliño, Departamento de Farmacología, Facultad de Medicina, Blasco Ibáez 15, 46010 Valencia (Spain); Phone: +34-96/386 4621; Fax: +34-96/3864972; E-mail: alino@uv.es

Key words: Gene transfer, gene delivery, liver, α-1 antitrypsin, genomic DNA, nonviral vector, fluid endocytosis, naked DNA, pig, catheter

Abbreviations: γ-glutamyl transferase, (GGT); albumin, (Alb), alanine aminotransferase, (ALT); alkaline phosphatase, (ALP); aspartate aminotransferase, (AST); human α-1 antitrypsin, (hAAT); matrix-assisted laser desorption ionization time-of-flight, (MALDI-TOF); plasmid completely digested with SalI, (pTG/SalI)

Received: 16 June 2005; Accepted: 01 July 2005; electronically published: July 2005

Summary

Success has been achieved with nonviral gene delivery to mouse liver, resulting in long-term therapeutic plasma levels of human α-1 antitrypsin (hAAT) protein, employing the hydrodynamic procedure. Now we contribute to explore the mechanism involved in the successful procedure, with the aim of circumventing the existing serious limitations for application to clinical practice. The results from mouse hydrodynamic gene transfer experiments support the following: a) the procedure mediates good dose-response efficacy, limited liver toxicity and long-term gene expression; b) the mechanism of gene delivery appears to be unspecific and involves transient inversion of intrahepatic flow, sinusoidal blood stasis and massive fluid endocytic vesicles in hepatocytes. In addition, since increased intrahepatic pressure induced by blood flow inversion could trigger hepatocyte DNA uptake, we have studied the efficacy of pig liver gene delivery by catheter-mediated retrodynamic injection of hAAT gene through the hepatic vein. The results show that in the same way as in mouse experiments: a) maximal plasma levels of human protein are observed on day 10-15 after injection, but the amount of protein is three orders of magnitude lower; b) maximal liver injury is observed two hours after retrodynamic perfusion, though it was very limited - suggesting that catheter perfusion is a mild procedure and/or that perfusion is limited to a very small region of the liver; c) electron microscopy shows the massive presence of large vesicles lining the membrane of hepatocytes alongside endothelial cells, though in contrast to the observations in mice, the vascular endothelium is very continuous and/or scarcely fenestrated.

I. Introduction

The liver is an organ with a high gene therapy potential (Strauss, 1994; Dawern, 2001), due to its many metabolic functions, large size and relatively easy access. The liver produces and secrets into the bloodstream many proteins with an important role in health, such as albumin, α-1-antitrypsin, coagulation factors, and fibrinogen. In addition, many disorders of liver metabolism are caused by monogenic defects. Since naked DNA is the simplest and safest gene delivery system (Herweijer and Wolff, 2003), multiple approaches have been attempted for *in vivo* liver gene delivery (Budker et al, 1996; Liu et al, 1999; Zhang et al, 1999; Liu and Huang, 2001a, b, 2002), though hydrodynamic transfection has become widely used (Ferber, 2001) because of its simplicity and high efficiency. On the other hand, human AAT deficiency is a good candidate for clinical and experimental gene therapy strategies and therefore, a broad variety of systems have been developed to transfer genes into the liver Employing the hydrodynamic procedure, high efficiencies of human α-1 antitrypsin gene transfer have been described (Zhang et al, 2000; Stoll et al, 2001), resulting in long-term therapeutic levels of human α-1-antitrypsin in mouse plasma (Aliño et al, 2003). Although the procedure has great limitations for application to clinical practice, the recently suggested mechanism involved in hydrodynamic liver mediated gene transfer (Aliño et al, 2003; Liu et al, 2004; Zhang et al 2004; Crespo et al 2005) offers opportunities to improve the technique in larger animals to

determine whether the efficacy of the procedure could be maintained under mild conditions as an established medical procedure. In the present work, we contribute to clarify the mechanism of hydrodynamic gene transfer efficacy to the liver in mice and, because of its potential application to clinical practice, we attempt to adapt the procedure to large animal models by catheter-mediated retrodynamic gene injection into the hepatic vein.

II. Material and methods

A. Animals

C57BL/6 mice (B&K Universal Ltd.) were used for all experiments. The animals were kept under standard laboratory conditions and housed 3-4 mice per cage. Pigs were obtained from a farm that works for Faculty of Medicine and maintained in individual pigsties. The experiments were approved by the Biological Research Committee of the Faculty of Medicine of the University of Valencia (Spain).

B. Reagents

Goat anti-hAAT antibodies (non-conjugated and peroxidase conjugated) were obtained from Sigma (SIGMA Chemical Co., Spain), and the immunohistochemical developing system LSAB from DAKO (Denmark). pTG7101 is a plasmid containing the full length of the human AAT. The plasmid was a generous gift from Dr. P. Meulien and Dr. J.P. Lecocq (Transgène S.A., Strasbourg, France). The TG7101-18.6 kb plasmid employed in the present work contains a 16.5 kb genomic fragment of hAAT gene, cloned in SalI site of Poly III-I plasmid. In addition, it contains a 1.8 kb genome sequence upstream promoter, the promoter and full length of human AAT gene, and 3.2 kb downstream gene.

C. *In vivo* gene transfer in mouse

For hydrodynamic liver gene transfer, C57BL/6 mice (n=5 per group) were injected into the tail vein with a volume of saline solution equivalent to 10% of body weight in 5 sec., containing different amounts of plasmid DNA, using a 3-ml latex-free syringe (Beckton Dickinson). For liver gene transfer by mechanical massage, mice were injected with 40 μg plasmid DNA in 200 μl saline (0.9% NaCl), 1 min. after manually massaging the liver as previously described (Liu and Huang, 2002). Blood samples (200 μl) were taken from the tail vein, using heparinized glass capillaries. After centrifugation, plasma was recovered and a pool was obtained mixing 50 μl/mouse for each group. The pooled plasma samples were kept at -20°C until ELISA assay and biochemical study of liver injury.

D. *In vivo* gene transfer in pig

Anesthesia of female pigs (25-30 kg) was induced with ketamine (5 mg/kg), meretomidine (10 mg/kg) and azapirone (2 mg/kg) and it was maintained with 2% propozole (10-15 ml/h). After femoral dissection, a balloon catheter was introduced and placed into the hepatic vein from the inferior cava vein. The location of the catheter was observed by X-ray imaging and the perfusion of Ultravist 370 contrast solutions (Iopramida, Shering). The catheter was fixed within the vein by balloon inflation, and then pTG 7101 plasmid in saline solution (20 μg/ml) was retrovenously injected (7.5 ml/sec.). Ten minutes later the catheter was removed. Blood samples were taken from ear veins and plasma was obtained as in the mouse experiments, and kept at -20°C until ELISA assay and biochemical study of liver injury. In some experiments the liver was retrovenously perfused with 2.5% glutaraldehyde saline solution, under conditions similar to those used for gene transfer, indicated above. Then the animals were sacrificed by KCl i.v. injection, the liver was removed, and after locating the catheter in the smaller intrahepatic branch of the hepatic vein, samples of tissue were obtained for histology studies.

E. ELISA of hAAT in mouse plasma

The hAAT in mouse plasma was quantified by ELISA, as previously described (Dasi et al 2001). In brief, goat anti-hAAT (0.2 μg/well) and goat anti-hAAT peroxidase conjugate (1.5 μg/well) were used as capture and detecting antibodies, respectively. The enzymatic reaction was induced with o-phenylenediamine (0.4 ng/ml, in citrate-phosphate buffer, pH 5) with 30% H_2O_2 (1.5 μl/ml). The reaction was stopped 2.5 min. later by adding 2M H_2SO_4.

F. In-gel enzymatic digestion and mass fingerprinting

The proteomic study for differential identification of hAAT versus mAAT protein in mouse plasma after hydrodynamic hAAT gene transfer was performed from excised protein band of interest in a Coomassie blue-stained SDS-10% polyacrylamide gel of mouse plasma. Analysis was carried out using an Applied Biosystems matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Voyager-DE mass spectrometer, operated in delayed extraction and reflectron mode, and the results were compared with the known trypsin digest protein databases (http://prospector.ucsf.edu/). In addition, the tryptic peptide mixture was analyzed by electrospray ionization in a QTrap (Applied Biosystems) instrument. The fragment pattern was interpreted by eye or using the on-line form of the MASCOT program at http://www.matrixscience.com.

G. Immunohistochemical staining of hAAT in mouse liver

Livers were fixed in 4% paraformaldehyde and embedded in paraffin. Immunostaining was carried out as previously described (Aliño et al, 2003). In brief, deparaffinized tissue sections were incubated with 4% bovine serum albumin in PBS (1 h, 37°C). Then, the tissue sections were incubated in antibody solution (1:1000 diluted rabbit anti-hAAT) at 37°C for 1 h. Detection was carried out using the peroxidase kit LSAB-2. Tissue was counterstained with hematoxylin before dehydration.

G. Intravital microscopy

Liver blood microcirculation was viewed as previously described (Crespo et al, 2005), using an inverted Zeiss microscope (Axiovert 135M) connected to a video enhancer-zoom lens system and a color CCD-video camera allowing real-time imaging. Images were recorded for further analysis. In a typical experiment, mice weighing 20 g were anesthetized and a catheter was introduced into the left femoral vein. After hydrodynamic injection into the vein (2 ml of saline solution in 5 seconds, containing 40 μg of pTG7101 DNA plasmid), the intrahepatic hemodynamic changes were recorded for at least 10-15 minutes.

H. Electron microscopy

Small tissue pieces were immersed in phosphate Sørensen buffer (pH 7.4) solution containing 2.5% glutaraldehyde. Some animals were previously treated by hydrodynamic injected via the tail vein with 2 ml of 2.5% glutaraldehyde. For transmission electron microscopy, multiple 1-mm^3 pieces of liver were routinely processed and embedded in Epox resin. Ultrathin sections, stained with uranyl acetate and lead citrate, were examined under a Jeol JEM-1010 electron microscope. For scanning electron microscopy, pieces of liver slices (2 mm thick)

were routinely processed. In brief, samples remained in 2.5% glutaraldehyde solution overnight at 4°C, and were postfixed in 1% aqueous osmium tetroxide. After dehydration in an ethanol series and critical point drying, the samples of liver were fractionated and mounted onto plate supports and then sputter coated with gold. The samples were viewed with a Hitachi S-4100 scanning electron microscope at 10 kV.

III. Results

A. Efficacy of liver gene transfer: hydrodynamic and mechanical liver massage procedures

Mice (n=5 per group) were hydrodynamic injected with a single dose of pTG7101 plasmid and with increased amounts of plasmid (from 0.6 to 320 μg/mouse) for each group. The amount of hAAT in mouse plasma on days +15 and +30 after hydrodynamic transfection was measured by ELISA. The results (**Figure 1A**) indicate that increased hAAT can be observed in plasma as a function of the administered dose. Maximal effect (2.5-3.5 mg/ml of hAAT) was detected with doses of 40 and 80 μg/mouse, and this higher dose mediated negative effects, since an inverse dose-effect relationship was present. To evaluate the efficacy of gene delivery to liver by mechanical massage, mice (n=5) were treated with 40 μg pTG7101 plasmid (in 200 μl of saline solution) one minute after liver massage, and the treatment was repeated every 15 days for 5 times. In addition, blood samples were taken every 12 days after DNA injection to measure AAT in mouse plasma. The results (**Figure 1B**) indicate that increased hAAT was detected as a function of the treatment cycle number. However, the maximal response was observed after three cycles of treatment, and additional cycles of treatment mediated negative effects. In addition, we must underscore that the maximal effect was three orders of magnitude lower than that achieved with the hydrodynamic procedure.

B. Long-term therapeutic hAAT gene expression by hydrodynamic procedure

In this experiment, mice were hydrodynamically treated with the maximal (80 μg) and supramaximal (320 μg) dose of pTG7101 plasmid, and 12 months later one additional dose of 80 μg of plasmid was administered. The amount of hAAT in mouse plasma two years after the first treatment is presented in **Figure 2**. The data show that the first treatment with 80 μg DNA results in therapeutic plasma levels of human protein (>0.9 mg/ml) for 6 months, after which the concentrations progressively decrease. However, a subtherapeutic plasma level of hAAT was observed from the first month of treatment with 320 μg DNA. In both cases, stationary subtherapeutic plasma levels of hAAT (26-36 μg/ml) were observed at the end of the first year of treatment, and a second hydrodynamic dose of 80 μg DNA mediated long-term therapeutic plasma levels of human protein during 8 months - the stationary subtherapeutic protein in plasma at the end of the second year being higher (72-183 μg/ml).

C. Mouse liver toxicity of hydrodynamic transfection

With the aim of evaluating liver injury with the hydrodynamic procedure, mice (n=5) were injected with 40 μg of pTG7101 plasmid, and blood plasma was collected from the tail vein at different timepoints. A pool of mice plasma was analyzed to evaluate the effect of treatment on the plasma levels of albumin (Alb), alanine aminotransferase (ALT), aspartate aminotransferase

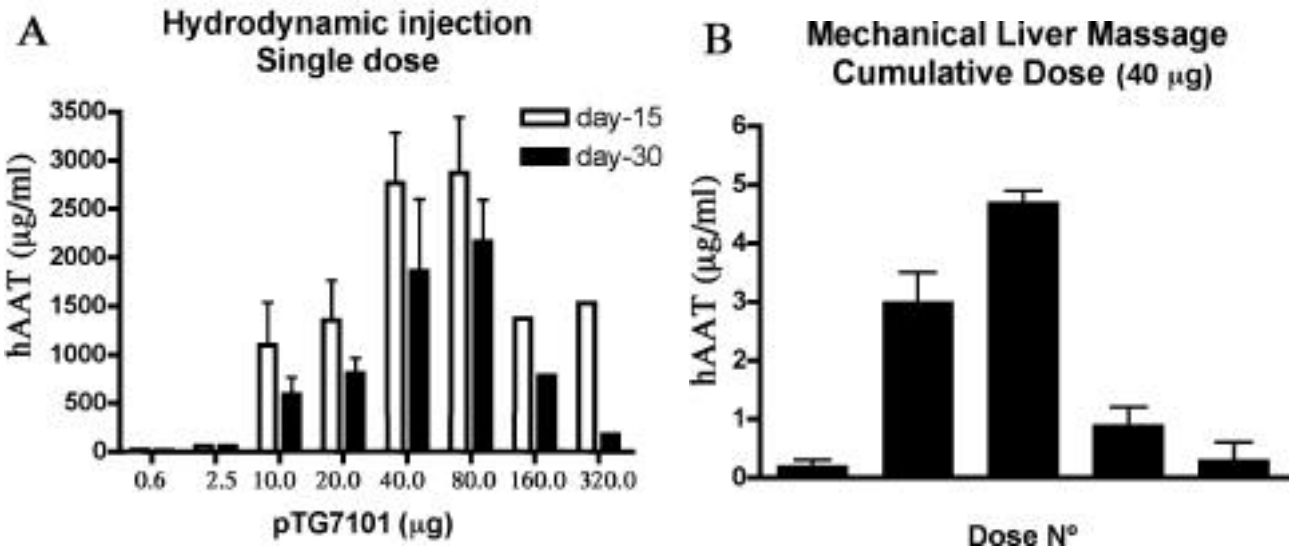


Figure 1. Efficacy of hAAT gene transfer. Mice (n=5 per group) were i.v. injected with plasmid pTG7101 containing the full genomic hAAT gene by two procedures: the hydrodynamic (**A**) and mechanical liver massage (**B**) employing independent single doses or cumulative doses, respectively. The human protein in mouse plasma was measured by ELISA on day 15 and also on day 30 in the hydrodynamic procedure.

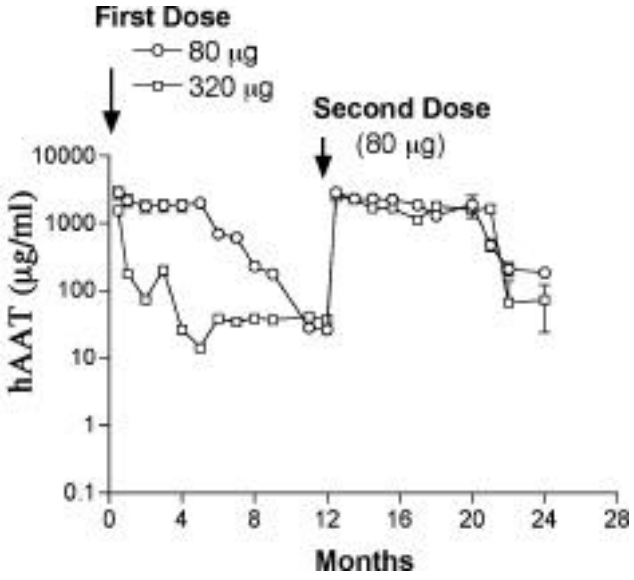


Figure 2. Effect of dose on long-term therapeutic plasma levels of hAAT in mouse plasma after hydrodynamic i.v. gene transfer.

(AST), γ-glutamyl transferase (GGT) and alkaline phosphatase (ALP). The results (**Table 1**) indicate that maximal liver injury occurred two hours after hydrodynamic injection, and that normal values were recovered two days later.

D. Identity of the transgene protein in mouse plasma

We have employed proteomic methodology to confirm the presence of hAAT in mouse plasma, previously quantified by ELISA. The MALDI-TOF peptide mass fingerprint shown in **Figure 3** allowed the unambiguous identification of 11 peptides of the mouse protein α-1-antitrypsin-1 precursor (SwissProt accession code P07758), with a MOWSE score of 2.81 e+005. Another three ions (at m/z 1642.0, 2648.5 and 3665.9) were compatible with theoretical fragments of the human α-1-antitrypsin protein (SwissProt accession code P01009). To confirm the identity of the mouse and human peptides, selected ions were subjected to collision-induced fragmentation. Thus, the sequence derived from the fragmentation pattern of the doubly-charged ion at m/z 821.8 (MALDI-TOF ion at m/z 1642.0), shown as an insert in **Figure 3**, was identified as ITPNLAEFAFSLYR. This amino acid sequence corresponds to the human α-1-antitrypsin protein at positions 50-63. As a whole, these data confirmed the presence of both the mouse and the human α-1-antitrypsin proteins in the plasma of the mice transfected with pTG-7101 plasmid.

E. Immunohistochemical staining of hAAT

Cells producing hAAT in mouse hepatocytes were identified by immunohistochemical sections of mouse liver (**Figure 4**). A low background was observed, since no unspecific stain was present in hepatocytes from control livers. However, liver sections from mice transfected with pTG7101 showed intense immunoreaction in some hepatocytes. Although has been described that hydrodynamic transfected cells are mainly located around central veins (Zhang et al 2000, Aliño et al 2003) we also observe stained hepatocytes in other distant territories.

Table 1. Mouse liver toxicity of hydrodynamic transfection

Time	Alb	ALT	AST	GGT	ALP
15 min	14	601	1201	20	170
30 min	18	760	1691	32	165
1h	16	1208	2335	36	149
2h	13	1501	2658	45	158
4h	17	1028	2385	32	124
1d	20	258	337	29	158
2d	23	62	86	8	128
5d	23	46	77	5	157
10d	23	45	360	15	155
15d	19	69	367	19	160
Normal values	25-48 g/l	26-77 U/l	54-269 U/l	< 6 U/l	45-222 U/l

Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGP, γglutamic aminotransferase; ALP, alkaline phosphatase
Each analysis was performed on the pooled plasma samples from five mice

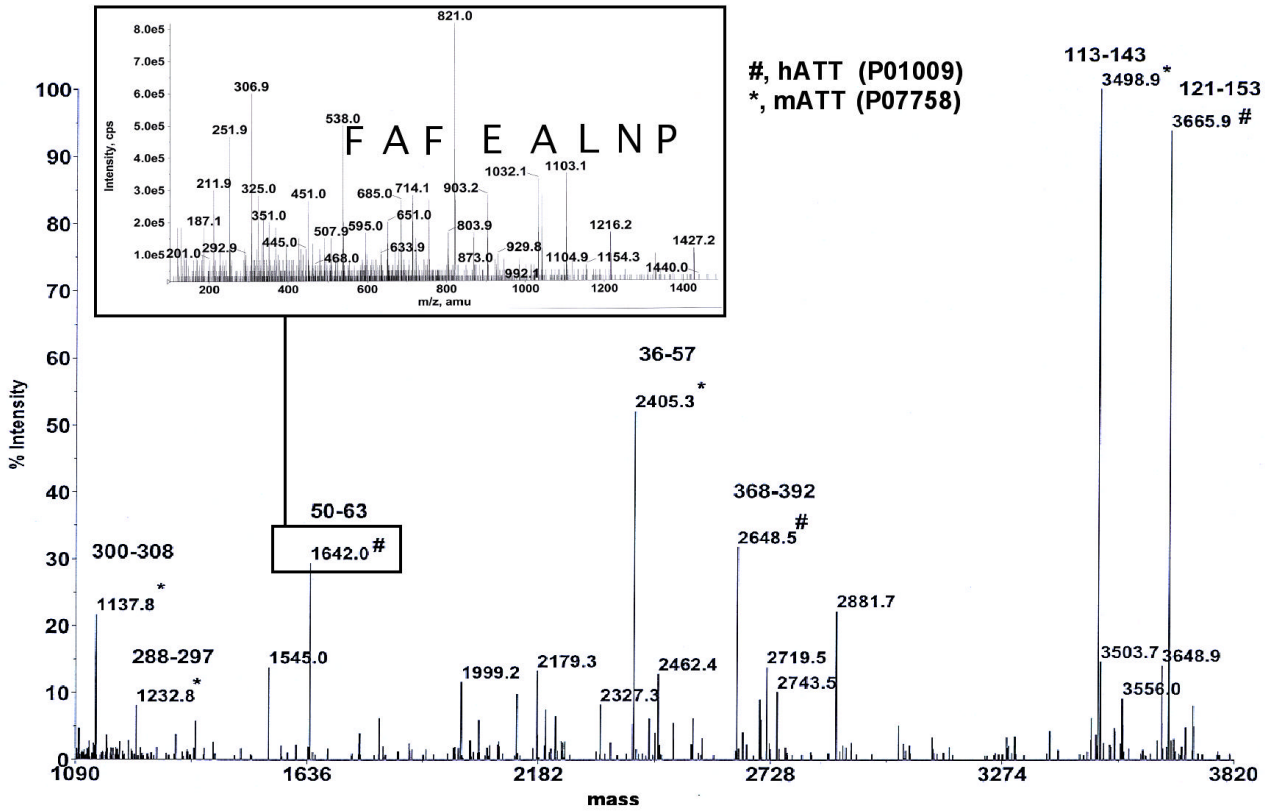


Figure 3. MALDI-TOF-MS spectrum of the in-gel tryptic digestion of the plasma α-1-antitrypsin band of mouse transfected with pTG-7101 plasmid. The monoisotopic masses of the tryptic peptide ions that matched the SwissProt database entries P07758 for the murine (*) and P01009 for the human (#) proteins are displayed. The corresponding protein sequence fragments are indicated above each assigned ion. Insert, CID-spectrum of the doubly-charged ion at m/z 821.8 (which corresponds to the MALDI-TOF ion at m/z 1642.0) showing a partial sequence assignment used to confirm the presence of the human α-1-antitrypsin transcript in the serum of the transfected mouse.

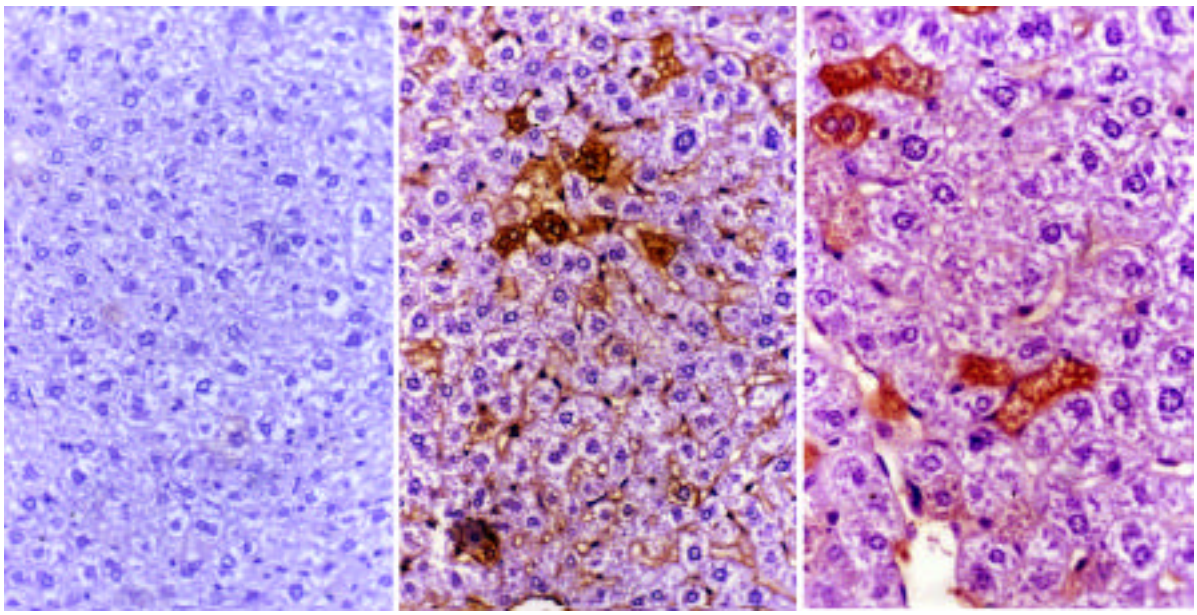


Figure 4. Immunohistochemistry of hAAT in mouse liver. Rabbit anti-human α-1 antitrypsin was used as primary antibody for all panels. Liver sections of non-transfected mice (left panel) shown no significant immunoreactivity, but specific immunostaining can be observed in liver sections from treated mice (one month before) with 20 μg pTG7101 plasmid.

G. Intravital microscopy of liver microcirculation

Hydrodynamic injection was performed through the left femoral vein (draining into the inferior cava). The aim of these experiments was to confirm whether the venous pressure inversion between the portal and inferior cava veins (draining into the inferior cava) after hydrodynamic injection could result in inverted hemodynamic flow into the liver. The results are summarized in **Figure 5**, where portal (P) and central (C) veins are identified, and the time course of the experiment (hours:minutes:seconds) is indicated. Before hydrodynamic injection (10:01:00), the image shows a liver area with a liver acinus in which the normal blood flow is from portal vein to central vein through many sinusoids. Liver edema was induced by hydrodynamic injection, and one minute later (10:02:00) we clearly differentiated two sinusoidal areas, one dark around the central vein in which blood flow circulation was stopped, and another light area around the portal vein in which blood flow was reduced but not stopped. Three minutes after hydrodynamic injection (10:04:00), blood stasis around the central vein remained, but blood circulation progressively recovered, and 3-4 min. later (10:10:00) most of the sinusoidal circulation was restored and only a few sinusoids remained without blood flow (dark sinusoids to the right of image).

H. Electron microscopy: hepatocyte morphological changes

A 2.5% solution of glutaraldehyde was hydrodynamically injected to avoid the tissue fixation time delay due to low fixative diffusion, and thus to observe the early morphological changes in hepatocytes after injection. **Figure 6** shows the more relevant morphological changes of liver tissue without (**Figure 6A**) and with (**Figure 6B and C**) mouse hydrodynamic injection, in which massive endocytic vesicles were observed in hepatocytes. Both transmission (**Figure 6B**) and scanning (**Figure 6C**) electron microscopy confirm, in livers of hydrodynamically injected mice, the presence of large vesicles lining the membrane of hepatocytes related with blood vessel endothelial cells, though in no case was hepatocyte plasma membrane disruption observed. In addition, the vascular endothelium was very discontinuous and largely fenestrated.

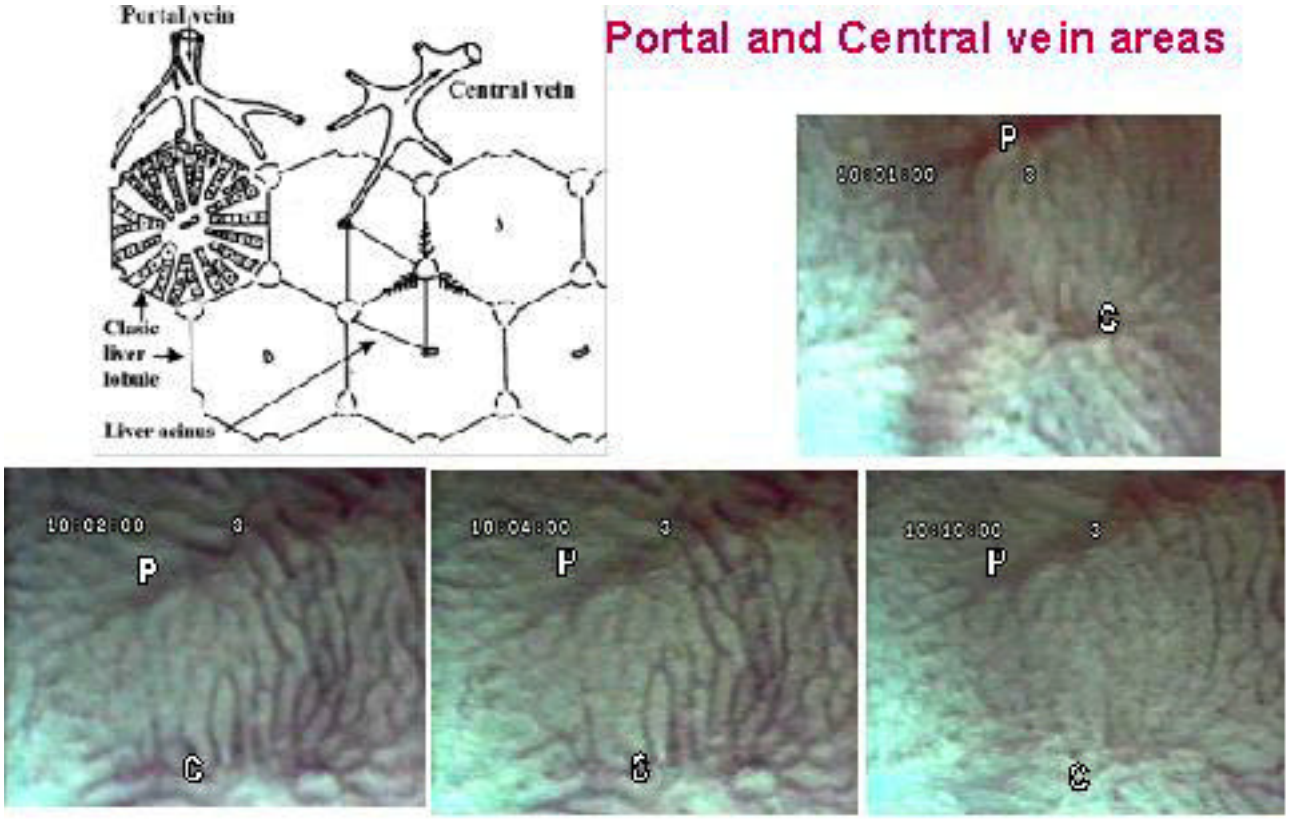


Figure 5. Intravital liver blood microcirculation. The in vivo observation of mouse liver blood flow was performed using an inverted microscope with a 20-fold objective. Mice were hydrodynamically injected in the left femoral vein and images including the portal vein (P) and central vein (C) of liver acinus were captured before (10:01:00) and 1, 3 and 9 min after injection (10:02:00, 10:04:00, 10:10:00). The direction of sinusoid blood flow was from portal vein to central vein and one minute after hydrodynamic injection (10:02:00) liver oedema was observed. In addition, it can be observed both light and dark sinusoid areas, corresponding to reduced (but nonstopped) and stopped sinusoidal blood flow, respectively. Nine minutes after injection, blood microcirculation was recovered (most sinusoids become light), although a few sinusoids remained without blood flow (dark sinusoids in the right of the image).

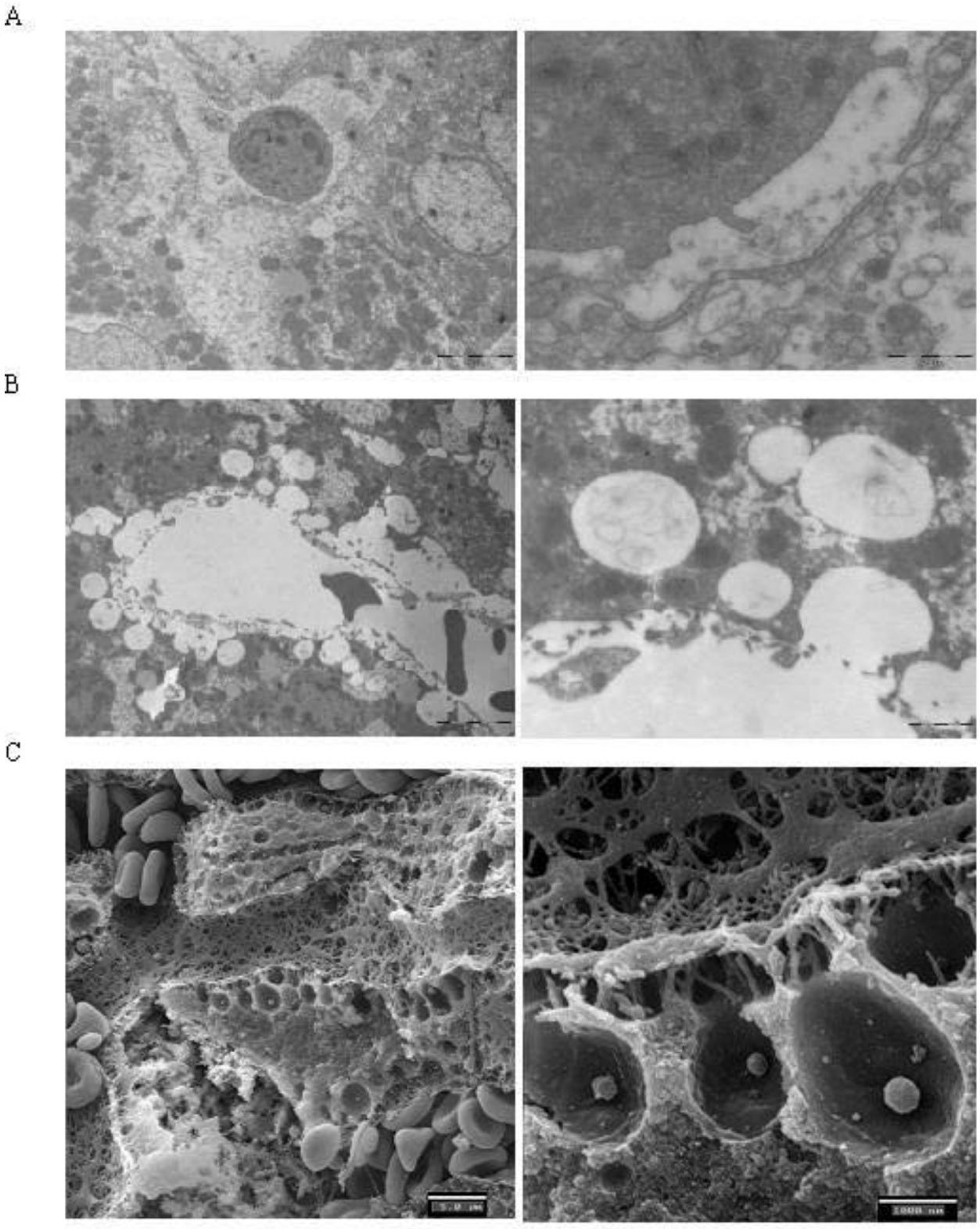


Figure 6. Mouse liver electron microscopy. Liver Transmission electron microscopy from control (**A**) and hydrodynamically injected mice with glutaraldehyde (**B**). In the control animal, a blood cell is placed inside a sinusoid (**A-left**), besides the continuous sinusoid endothelium under which the membrane of a hepatocyte can be observed (**A-right**). Transmission and scannig electron microscopy images from hydrodynamically injected mice (**B and C, respectively**) show the expansion of sinusoid lumen, the breaking of continuous endothelium and the presence of massive (**B and C-left**) and large endocytic vesicles (**B and C-right**) in the hepatocytes.

I. Nonspecific process of gene transfer to hepatocyte

In order to determine whether any plasmid region is implicated in hydrodynamic gene transfer by a receptor mediated mechanism, 40 μg of pTG7101 plasmid, with the ability to induce 80% Emax, was co-injected with an equal dose (40 μg) of plasmid completely digested with SalI (pTG/SalI) or XhoI (pTG/XhoI). SalI digestion results in two DNA fragments containing the complete hAAT gene and the poly-III vector, whereas digestion with XhoI results in 5 fragments with hAAT gene disruption. The results (**Figure 7**) indicate that: a) Similar efficacy was achieved using the complete circular or SalI digested plasmid, and that increased plasma levels of hAAT could be obtained by including an additional 40 μg of circular plasmid in the co-injection; b) The co-injection of pTG7101 plasmid with pTG/XhoI fragments (pTG(40)/SalII+pTG(40)) did not inhibit transfection efficacy, and an increased dose of circular plasmid likewise increased the efficacy. If a specific cell surface receptor were involved in DNA binding, gene transfer should be expected to suffer inhibition at least in the pTG/XhoI co-injection. The levels of gene expression were not modified, however, thus suggesting that hydrodynamic gene transfer to hepatocytes does not involve a receptor-mediated process. Consequently, a non-specific process must be involved.

J. Pig liver efficacy and toxicity of catheter mediated hAAT gene delivery

The balloon catheter was introduced through the femoral vein into the hepatic vein, under X-ray imaging and injections of small volumes (3-5 ml) of Ultravist 370 contrast solutions. Then the balloon was fixed by inflation and 100 ml of a saline solution of 20 μg/ml pTG7101 plasmid was retrodynamically perfused at a rate of 7.5 ml per second. Then blood samples were taken at different timepoints. **Figure 8** shows the results of ELISA analysis of hAAT in pig plasma (n=3). In a way similar to mice, the maximal plasma levels of human protein were observed on day 10-15 after injection, though the amount of protein was three orders of magnitude lower.

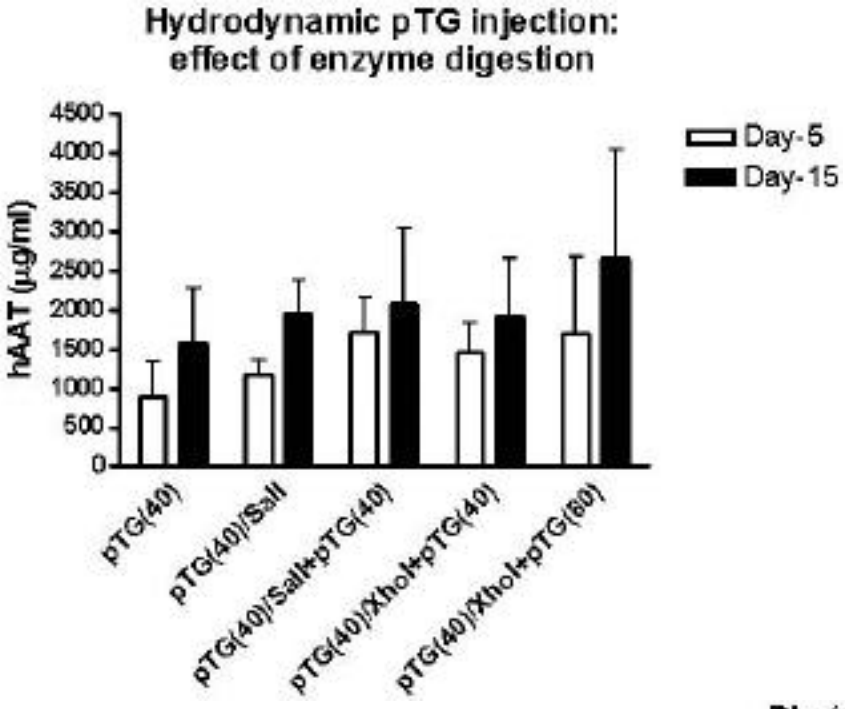


Figure 7. Effect of the co-injection of undigested and digested pTG7101 plasmid (using restriction enzymes). Groups of mice (n=5) were treated with 40 μg of undigested (pTG), SalI digested plasmid in the absence (pTG/SalI) or presence (pTG/SalI+pTG40) of 40 μg of additional circular undigested plasmid. The effect of XhoI digested plasmid in the presence of 40 or 80 μg of circular undigested pTG7101 plasmid (pTG/XhoI+pTG40 and pTG/XhoI+pTG80, respectively) is also showed. The hAAT protein in plasma, on days 5 and 15 after hydrodynamic transfection,, was measured by ELISA.

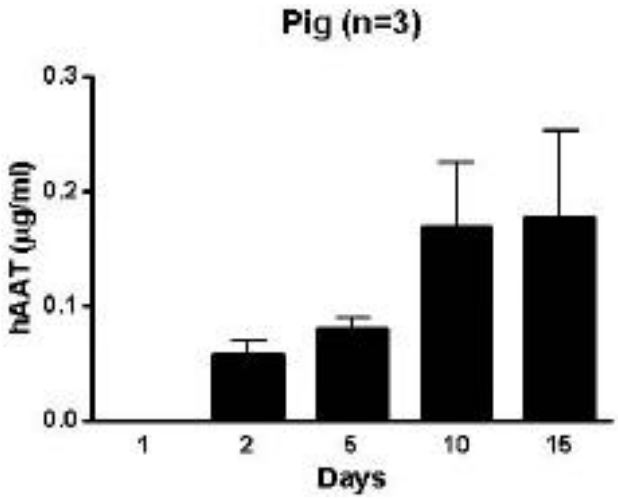


Figure 8. Effects of catheter-mediated hAAT gene transfer to pig liver. Saline solution containing 20 μg/ml of pTG7101 plasmid was retrodynamically injected (100 ml) into hepatic vein at 7.5 ml/second ratio. The levels of hAAT in pig plasma were measured by ELISA.

The biochemical plasma study of liver injury induced by the catheter-mediated procedure, is summarized in **Table 2**. As in the mouse experiments after hydrodynamic injection, maximal liver injury was observed two hours after retrodynamic perfusion, but was very limited - suggesting that catheter perfusion is a mild procedure and/or that perfusion is limited to a very small region of the liver.

K. Pig liver electron microscopy

A 2.5% solution of glutaraldehyde was retrodynamically perfused (100 ml at 7.5 ml per second) to observe the early morphological changes in hepatocytes. **Figure 9** shows the more relevant morphological changes in hepatocytes, in which massive endocytic vesicles were observed as well as the presence of large vesicles lining the membrane of hepatocytes related with blood vessel endothelial cells. In contrast to the situation in mice, however, the vascular endothelium was very continuous and/or scarcely fenestrated.

IV. Discussion

We have observed (Crespo et al, 1996; Dasi et al, 2001) that long-term expression and stable plasma levels of hAAT can be achieved by intravenous injection of a plasmid containing the human genomic gene controlled by the natural promoter, though the efficacy was four orders of magnitude lower than that required to mediate a therapeutic effect. On the other hand, it has been demonstrated (Liu et al, 1999; Ferber, 2001) that naked DNA can be efficiently transfected into the liver via a rapid i.v. injection of plasmid in a large volume solution (hydrodynamic procedure). Based on this procedure, therapeutic but very transient (Zhang et al, 2000)) or stable but subtherapeutic (Stoll et al, 2001) plasma levels of hAAT have been achieved in mice, and recently we have obtained long-term therapeutic plasma levels of hAAT (Aliño et al, 2003) employing a plasmid containing the genomic hAAT gene and the hydrodynamic gene transfer procedure. The present study contributes to clarify the mechanism involved in the success of hydrodynamic liver gene transfer, with the aim of adapting the procedure to large animals, with a potential for clinical application.

Nucleic acid transfer to the liver can be performed with high efficiency by means of methods that induce an increase in intrahepatic pressure. In this sense, we have comparatively evaluated the efficacy of the hydrodynamic procedure versus mechanical massage, showing that the former approach affords the highest efficacy in terms of greater plasma levels of human α-1 antitrypsin protein in blood after transfection. Interestingly, we observed a dose-dependent response in both cases, with a maximum effect after the administration of 40-80 μg/mouse of pTG7101 plasmid. Higher doses resulted in lower protein levels in blood, indicating that such doses are less effective. Although both procedures probably mediate their effects through similar mechanisms, based on an increase in intrahepatic pressure, a substantial difference must exist that makes the hydrodynamic procedure much more efficient when using the same dose. For this reason we continue to explore the mechanisms implicated in the high efficiency of gene transfer with the hydrodynamic procedure.

With the aim of defining the clinical usefulness of the hydrodynamic procedure, we studied the efficacy of gene expression over time, and its toxicity following administration. For the first issue we performed a study with two doses: one maximal and the other supramaximal. Interestingly, the maximal dose expressed a therapeutic level of the protein during long time periods (>6 months), after which the levels started to decrease until stabilization was reached (30 μg/ml approx.) 12 months later. In contrast, the supramaximal doses of the plasmid (320 μg/mouse) expressed subtherapeutic plasma levels of the human protein, reaching after twelve months the same levels as in the first case. After this first year, a second dose of 80 μg of plasmid restored the therapeutic plasma levels of the protein for a longer period than that observed with the first dose, and also the stabilized levels at the end of the second year were higher (100 μg/ml). These data suggest that the procedure is well tolerated, as it allows potentially therapeutic responses during long time periods, and repeated doses do not decrease efficacy. In order to determine to what extent the effects are generated in the first moments after administration, we evaluated hepatic toxicity by measuring liver injury enzymes, confirming that maximum hepatic toxicity is reached in the first two hours after hydrodynamic injection. The levels of these enzymes normalized in the following 24-48 hours. These data indicate that the toxic effects are generated in the first moments after transfection.

Table 2. Pig liver toxicity of catheter mediated transfection

Time	Alb	ALT	AST	GGT
0h	25	47	58	46
2h	26	71	123	47
1d	26	52	70	45
5d	24	63	67	50
10d	25	59	78	46
15d	23	49	57	49
Normal values	22-40 g/l	21-46 U/l	15-55 U/l	10-52 U/l

Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGP, γglutamic aminotransferase; ALP, alkaline phosphatase

Each analysis was performed on the pooled plasma samples from five mice

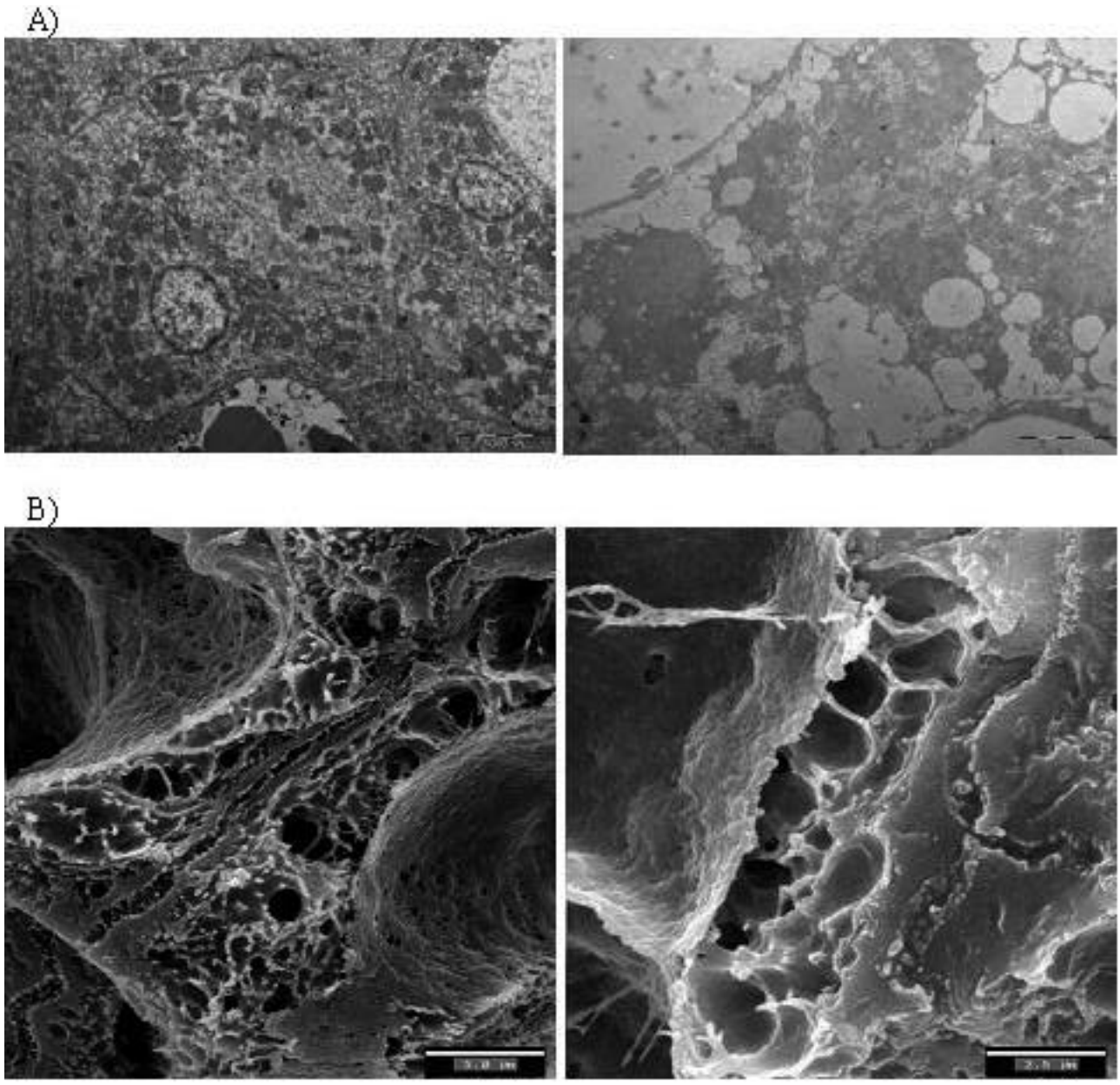


Figure 9. Pig liver electron microscopy. Transmission electron microscopy of liver from control (**A-left**) and catheter-mediated glutaraldehyde perfusion (**A-right**). Scanning electron microscopy of catheter mediated perfused liver is shown in **B**. massive and large endocytic vesicles lining the hepatocytes beside the endothelial cells were observed only in treated animals but not in controls. The vascular endothelium was very continuous and scarcely fenestrated.

Once we confirmed that the hydrodynamic procedure generates a response of therapeutic interest, we aimed to confirm by other methods the presence of the human protein in mouse plasma. We employed two procedures based on proteomics and immunohistochemistry. In the first case, we could differentially identify both the human and the mouse proteins in the plasma of treated mice, and could also prove that the fragment masses for both proteins were of similar magnitudes - which strongly supports that both proteins were present at similar concentrations in plasma, as our quantitative ELISA assays previously had shown.

Finally, immunohistochemistry allowed us to confirm the presence of the human protein in mouse hepatocytes, with a preferential distribution in the areas around the central veins and also between the sinusoids located among the central and portal veins of the liver lobule.

Hydrodynamic transfer implies duplicating the circulation volume in the inferior cava vein during a very short time period (5 seconds approx.). In this way, the pressure of the suprahepatic vein reaches much higher levels than normal, which causes an inversion in blood circulation from the hepatic to the portal vein through the liver. Our previous studies illustrated this retrovenal process by means of intravital microscopy in the area of the central vein, confirming the existence of venous stasis in this territory. However, no previous observation of the blood flow in the complete liver lobule had been made, in which both the flow of the portal and central veins is observed simultaneously. Now we can demonstrate that hydrodynamic injection results in retrovenous blood flow

from the central to the portal vein in the first moments and in the large vessels, with venous stasis mainly in the area of the central vein. On the other hand, however, blood flow, slows down but does not stop completely in the periportal sinusoids. The pericentral vein stasis is sustained during some minutes, followed by a gradual restoration of flow 9-10 minutes after the injection. As we have previously reported that gene transfer occurs mainly in the pericentral venous areas, we now offer complementary information indicating the absence of venous stasis in the periportal area - which could also be the cause of the small number of transfected cells in this liver territory.

With the aim of studying the morphological changes following hydrodynamic injection, mice were treated with this procedure, injecting a saline solution of 2.5% glutaraldehyde in order to instantaneously fix the morphological changes produced in the first moments after injection (Crespo et al, 2005). By means of transmission and scanning electron microscopy, we could observe the presence of a great number of large vesicles in the cell membranes of the hepatocytes close to the endothelial cells of the sinusoids, which in turn showed very evident discontinuity. These images contrast with those obtained in the control group, where no dilatation of the sinusoid was observed, and the spaces or endocytosis vesicles were very limited. Scanning electron microscopy also showed the presence of a highly fenestrated endothelium, and the surface of the surrounding hepatocytes showed an abundance of very large endocytosis vesicles that could be directly related to the process of DNA internalization. There were also endocytosis vesicles in the liver after mechanical massage (data not shown), suggesting that apart from vesicle formation, the input of a minimum hydric volume is required for transfection to be effective. In this sense, some authors have reported that the expression efficiency of a gene injected into the cellular cytoplasm is directly proportional to the hydric volume in which this gene is administered (Zhang et al, 2004). This results in the fact that the larger the accompanying hydric volume reaching cytoplasm, the greater the efficacy - thus suggesting that a threshold volume must exist above which the hydrodynamic process affords optimized transfection efficacy.

However, DNA entrance by massive endocytosis does not discard the fact that DNA could enter cells bound to specific receptors on the cell surface, and does not rule out the possibility that in some way the hydrodynamic procedure could facilitate the internalization of both molecules: DNA in solution and DNA associated to the surface receptors. We found that enzymatic digestion of the plasmid with Sal I, which removes the complete gene from the plasmidic vector, does not inhibit transfection efficacy. Indeed, our data suggest that it could even increase such efficacy. These findings support that there should not be sequences in the extragenic region of the plasmidic vector which would be limiting gene internalization by the hepatocytes. With the purpose of confirming this point, we performed plasmid digestion with Xho I, which removes the complete gene in the same way as Sal I, but also splicing the gene itself at several points. When the circular plasmid is administered together with an equivalent quantity of Xho I-digested plasmid, we found no modification in gene expression efficacy. Moreover, double circular plasmid dosing increases such efficacy, suggesting that the gene fragments do not interfere with plasmid entry into the hepatocytes. In this sense, our results support that gene entry into the hepatocytes occurs via an unspecific mechanism, though we cannot exclude the possibility that part of the phenomenon may be of a specific nature.

The results obtained support the idea that gene transfer by means of the hydrodynamic procedure is carried out by an unspecific mechanism in which the increase in intrahepatic pressure, due to the injection, promotes massive endocytosis that generates intracellular hydric movement which, after reaching a certain threshold level, facilitates gene entry. Considering that this procedure is not suitable for clinical application, because of the severe systemic hydrodynamic changes involved, we contemplated the possibility of designing a similar model in larger animals. We required a gene transfer procedure by means of a selective intrahepatic pressure increase. To this effect we used pigs in which a catheter was advanced through the femoral vein to reach the inferior cava vein and then the suprahepatic vein, where the catheter was fixed with a balloon to avoid retrograde displacement. In this way we retrodynamically injected towards the liver and through the hepatic vein, 100 ml of the plasmid in saline solution (20 μg/ml), at a rate of 7.5 ml/second. Posteriorly, the animals recovered and their blood samples were used to evaluate transfection efficacy and liver toxicity. The efficacy results showed the response over time to be similar to that observed in mice, since the maximum levels were reached 10-15 days after transfection. However, the plasma levels of the human protein were four order of magnitude lower (30 μg/ml) than those reached in the murine model. Curiously, the procedure was well tolerated and, as in mice, the maximum toxicity was reached two hours after perfusion - suggesting that the mechanism of local gene transfer mediated through the catheter procedure must be similar to the hydrodynamic procedure in mice. To confirm this, we conducted additional experiments to determine whether we could observe the morphological changes seen in mice after gene infusion, in pig hepatocytes. Transmission and scanning electron microscopy confirmed that in the perfused area the hepatocytes exhibited morphological changes equivalent to those induced in mice by hydrodynamic injection, i.e., a massive presence of megavesicles lining the cells in contact with the vascular endothelium, while the areas related to the bile canaliculi were vesicle-free. It should be pointed out that the endothelial changes were not severe - exhibiting areas with a continuous structure and others with a medium fenestrated organization but no venous pressure was measured during gene delivery. The last aspect, should be take considered for the future research because it could contribute to determine the final efficacy of the procedure. Although the catheter was introduced under X-ray guidance, and its location was confirmed by contrast injection, its exact situation was confirmed after perfusion

with glutaraldehyde and sacrifice of the animal. When the organ was extracted we verified that the true perfused area comprised only a small portion of the total liver and the morphological changes of hepatocytes was studied in samples from the smaller liver area than was macroscopically identified as well perfused. Therefore, we think that the limited efficacy of the procedure could be related first to the absence of important changes in endothelial permeability - which could limit gene diffusion through the endothelium - and secondly to the small liver mass and/or volume transfected. Considering all these points, we consider improving the procedure to transfect a larger area of the organ and also to increase endothelial discontinuity. This could improve transfection efficacy, making the procedure an excellent candidate for clinical application.

Acknowledgments

This work has been supported by the University of Valencia, and partially by Ministerio Educacion y Ciencia SAF2004-08161 and Generalitat Valenciana GV04B/179 projects (Spain). We thank Dr. Juan Sanchis, Ximo Gil and Rafael Sanjuan from Hospital Clínico Universitario de Valencia for his valuable catheterism support.

References

Aliño SF, Crespo A, Dasí F (**2003**) Long-term therapeutic levels of human α-1-antitrypsin in plasma after hydrodynamic injection of nonviral DNA. **Gene Ther** 10, 1672-1679.

Budker V, Zhang G, Knechtle S, Wolff JA (**1996**) Naked DNA delivered intraportally expresses efficiently in hepatocytes. **Gene Ther** 3, 593-598.

Crespo A, Peydro A, Dasi F, Benet M, Calvete JJ, Revert F, Aliño SF (**2005**) Hydrodynamic liver gene transfer mechanism involves transient sinusoidal blood stasis and massive endocytic vesicles. **Gene Ther** 12, 927-935.

Crespo J, Blaya C, Crespo A, Aliño SF (**1996**) Long-term expression of the human α1-antitrypsin gene in mice employing anionic and cationic liposome vectors. **Biochem Pharmacol** 51, 1309-1314..

Dasív F, Benet M, Crespo J, Crespo A, Aliño SF (**2001**) Asialofetuin liposome-mediated human α1-antitrypsin gene transfer *in vivo* results in stable long-term gene expression. **J Mol Med** 79, 205-212.

Davern T J (**2001**) Molecular therapeutics of liver disease. **Liver Dis** 52, 381-414.

Ferber D (**2001**) Gene therapy, safer and virus-free? **Science** 294, 1638-1642.

Herweijer H, Wolff JA (**2003**) Progress and prospects, naked DNA gene transfer and therapy. **Gene Ther** 10, 453-458.

Kawatava K, Takakura Y, Hashida M (**1995**) The fate of plasmid DNA aflter intravenous injection in mice, involvement of scavenger receptors in its hepatic uptake. **Pharm Res** 12, 825-830.

Liu F, Huang L (**2001a**) Improving plasmid DNA-mediated liver gene transfer by prolonging its retention in the hepatic vaculature. **J Gene Med** 3, 569-598.

Liu F, Huang L (**2001b**) Electric gene transfer to the liver following systemic administration of plasmid DNA. **Gene Ther** 9, 1116-1119.

Liu F, Huang L (**2002**) Noninvasive gene delivery to the liver by mechanical massage. **Hepatology** 35, 1314-1319.

Liu F, Lei J, Vollmer R, Huang L (**2004**) Mechanism of liver gene transfer by mechanical massage. **Mol Ther** 9, 452-457.

Liu, F, Song, Y.K, Liu, D (**1999**) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. **Gene Ther** 6, 1258-1266.

Moret I, Esteban J, Guillem VM, Benet M, Revert F, Dasí F, Crespo A, Aliño SF (**2001**) Stability of PEI-DNA and DOTAP-DNA complexes, effect of alkaline pH, heparin and serum. **J Control Release** 76, 169-181.

Skehel JJ (**1992**) Amantadine blocks the channel. **Nature** 358, 110-111 .

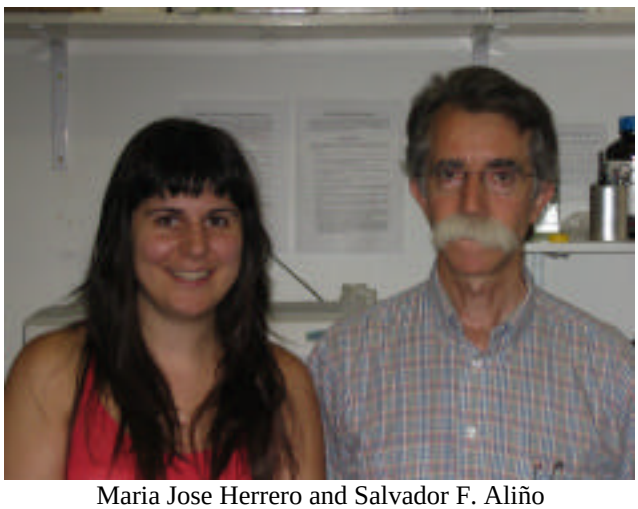
Stoll SM, Sclimenti CR, Baba E, Meuse L, Kay MA, Calos MP (**2001**) Epstein-Barr virus/human vector provides high-level, long-term expression of α(1)-antitrypsin in mice. **Mol Ther** 4, 122-129.

Strauss M (**1994**) Liver-directed gene therapy, prospects and problems. **Gene Ther** 1, 156-164.

Zhang G, Budker V, Wolf JA (**1999**) High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. **Hum Gene Ther** 10, 1735-1737.

Zhang G, Gao X, Song YK, Vollmer R, Stolz DB, Gasiorowki JZ, Dean DA, Liu D (**2004**) Hydroporation as the mechanism of hydrodynamic delivery. **Gene Ther** 11, 675-682.

Zhang G, Song YK, Liu D (**2000**) Long-term expression of human α1-antitrypsin gene in mouse liver achieved by intravenous administration of plasmid DNA using a hydrodynamics-based procedure. **Gene Ther** 7, 1344-1349.



Maria Jose Herrero and Salvador F. Aliño