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Original Paper

A Surgical Model for Isolating the Pig Liver in vivo for Gene Therapy

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Abstract

Several studies report results that suggest the need of vascularization blocking for efficient gene transfer to the liver, especially in nonviral gene therapy. In this study, we describe a surgical strategy for in vivo isolation of the pig liver, resulting in a vascular watertight organ that allows the evaluation of several gene injection conditions. The hepatic artery and portal, suprahepatic and infrahepatic cava veins were dissected. Then, liver vascularization was excluded for 5–7 min. In that time, we first injected 200 ml saline solution containing the p3c-eGFP plasmid (20 μg/ml) simultaneously through two different catheters placed in the portal and cava veins, respectively. Vital constants were monitored during the surgery to assess the safety of the procedure. Basal systolic/diastolic blood pressures were 92.8/63.2 mm Hg and dropped to 40.7/31.3 mm Hg at the end of vascular exclusion; the mean basal heart rate was 58 bpm, reaching 95 bpm when the blood pressure was low. Oxygen saturation was maintained above 98% during the intervention, and no relevant changes were observed in the ECG tracing. Peak plasma AST (aspartate aminotransferase) and ALT (alanine aminotransferase) levels were observed after 24 h (151 and 57 IU, respectively). These values were higher, but not relevant, in 60 ml/s injection than in 20 ml/s injection. Efficiency of gene transfer was studied with simultaneous (cava and portal veins) injection of eGFP gene at flow rates of 20 and 60 ml/s. Liver tissue samples were collected 24 h after injection and qPCR was carried out on each lobe sample. The results confirmed the efficiency of the procedure. Gene delivery differed between 20 ml/s (9.9–31.0 eGFP DNA copies/100 pg of total DNA) and 60 ml/s injections (0.6–1.1 eGFP DNA copies/100 pg of total DNA). Gene transcription showed no significant differences between 20 ml/s (15,701.8–21,475.8 eGFP RNA copies/100 ng of total

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Eur Surg Res 2013;51:47–57
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RNA) and 60 ml/s (12,014–36,371 eGFP RNA copies/100 ng of total RNA). The procedure is not harmful for animals and it offers a wide range of gene delivery options because it allows different perfusion ways (anterograde and retrograde) and different flow rates to determine the optimal conditions of gene transfer. This strategy permits the use of cell therapy and viral or non-viral liver gene therapy, especially appropriated to a wide variety of inherited or acquired diseases because of the liver's ability to produce and deliver proteins to the bloodstream.

Introduction

Most circulating serum proteins are synthesized within the liver and then delivered to the bloodstream where they are shipped to their specific site of action. There is a group of diseases in which the pathology is caused by a fault in a single protein, coded by a single gene. This fault causes the protein encoded by this gene to be wrong, affecting to its correct function and developing the illness. Gene therapy is a type of treatment that aims to use genes as drugs, and offers a huge therapeutic potential that has barely been exploited in routine clinical practice. Gene therapy can play an important role [1] in the treatment of these diseases because the correct gene encoding the adequate protein could be transferred to where the defective protein is produced and improve the pathology. These features make the liver an optimal target [2] for gene therapy. Its size and protein synthesis ability would empower the liver [3] to produce physiologic and/or therapeutic molecules usually formed elsewhere. Determining an efficient systematic model of gene transfer would open a wide range of possibilities, such as transferring therapeutic genes to patients with a deficiency in the expression of such genes [4], silencing genes in patients with upregulated gene expression [5], or developing a strategy [6] to treat patients with a viral infection. These different strategies allow the covering of a wide variety of diseases. This broad spectrum of possibilities underscores the capital importance of developing a systematic, efficient and safe model of gene transfer which would permit us to step up to the clinical setting in humans.

Extensive experience has been gained in nonviral gene therapy since gene transfer was demonstrated to be safe and efficient in mice with the injection of a large volume of plasmid solution (hydrodynamic) via the caudal vein [7, 8]. Therapeutic plasma levels (>1 mg/ml) of human α_1-antitrypsin protein were reached for longer than 6 months [9]. These results led the scientists to conduct their studies to develop a similar model applicable to larger animals more similar to humans, such as pigs. Several approaches [10–12] of noninvasive catheter-mediated retrovenous injection in individual liver lobes were evaluated. Results of these studies demonstrated effective gene transfer but much lower as compared with the murine model. This poor efficiency was a consequence of the low pressure reached within the liver. This may have been due to the high elasticity of the liver tissue, as deduced from studies of efficient hydrodynamic gene transfer in less elastic tissues such as cardiac tissue in pigs [13], or could have been due to outflow of the injected solution into other vessels. Sawyer et al. [14] demonstrated that an in vivo pressurized liver model is required in order to reach an intrahepatic pressure high enough for efficient gene transfer [15]. This idea has been confirmed in a study [16] in which a high expression of eGFP protein was reached in the pig liver through balloon-catheter retrovenous injection in individual liver lobes following vascular exclusion. Kamimura et al. [17] adopted a similar approach and reported high levels of luciferase activity taking into consideration the obstruction of outflow. Protein expression occurs mainly in the perfused area. Our group [18] followed this line, employing watertight human liver segments. The latter were obtained from surgical resection, and consequently

Eur Surg Res 2013;51:47–57
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resulted in vascular watertight tissue. Retrovenous ex vivo catheter-mediated injection of an eGFP gene solution was carried out, and gene expression data showed high efficiency of the transfer model. In summary, results suggest that retrograde injection and outflow blockade is needed for efficient gene transfer. In the present study, we describe a safe surgical model for the in vivo obtainment of watertight whole liver allowing anterograde, retrograde or simultaneous injection of therapeutic solution. This model allows analysis and comparison of system efficiency under different conditions and offers potential clinical applications, since it represents standardization of safe and efficient in vivo gene transfer in the liver – the latter being the most appropriate organ, since it is the main producer of serum proteins for the entire body.

Material and Methods

Animals

Female pigs were obtained from a farm working for the Health Research Institute (IIS) of La Fe Hospital (Valencia, Spain), and were housed in individual pigsties. The experiments were approved by the Animal Biological Research Ethics Committee of the Hospital La Fe.

Anesthesia of the pigs (18–23 kg) was induced with ketamine (Imalgene 100, Merial, France; 5–10 mg/kg i.m.), midazolam (Hospira 1 mg/ml, Madrid, Spain; 0.3 mg/kg i.m.) and propofol (Lipuro 2%, Braun, Melsungen, Germany; 4–6 mg/kg i.v.), and was maintained with sevoflurane (Sevorane, Abbott Laboratories, Madrid, Spain; 2.5%, inhalatory). Muscle relaxation was induced with vecuronium bromide (Norcuron 10 mg; 0.08 mg/kg i.v.). Morphine (0.4 mg/kg i.v.) was administered for intraoperative analgesia and buprenorphine (Buprex, Schering-Plough, Madrid, Spain; 0.02 mg/kg i.v.) for postoperative analgesia. Vital constants were monitored throughout the intervention. The pigs were sacrificed 24 h after the operation using potassium chloride (Braun 2 mEq, 20 mEq i.v.) after sedation. Blood samples (2 ml) were collected from an ear vein at 0 h (before plasmid injection), 1 h after injection and 24 h after injection before sacrifice. The liver was extracted and representative tissue samples of each lobule were collected for further analysis.

Surgical Equipment

Surgical laparotomy equipment, vessel loops (DEVON™, Covidien) for referencing vascular structures and clamps were required. We used three types of sutures, Biosyn™ 5/0 (Covidien) for vena cava, Prolene™ 6/0 (Ethicon) for portal vein, and Dexon™ 2/0 (Covidien) for closing the abdominal wall. Staples were used to close the skin.

Plasmid Construction

The plasmid p3c-eGFP (6.45 kb), containing the enhanced green fluorescent protein complementary DNA (cDNA) driven by pCMV (cytomegalovirus) promoter, was constructed by cloning eGFP into the *Hin*dIII site of pcDNA3 (Invitrogen, Barcelona, Spain), excised from peGFP-N1 (4.7 kb) plasmid vector (Clontech Laboratories, Saint-Germain-en-Laye, France).

In vivo Gene Transfer to Pig Liver

Two 14-Fr catheters (Bicakcilar, Istanbul, Turkey) were placed in the cava and portal veins for simultaneous gene transfer. After catheter fixation with needle and thread, a solution containing the eGFP plasmid (20 μg/ml) was injected with a high volume pump at different flow rates and a volume of 400 ml (approx. 1/1 of the organ weight according to body weight). Tissue samples were obtained from perfused areas. Tissue samples <1 mm in thickness were subsequently collected under sterile conditions and stabilized in RNAlater solution (Ambion). Samples were removed 1 day after collection and processed for molecular analysis to evaluate delivery efficiency and gene expression.

Quantitative PCR and RT-PCR

Two samples were taken from each every four liver lobes. Tissue samples were cut into small pieces and homogenized in RLT buffer (Qiagen®, Barcelona, Spain) with an Ultra-Turrax homogenizer (Hielscher Ultrasonics GmbH, Teltow, Germany). Further purification with the RNeasy midi kit (Qiagen) was performed before spectrophotometric quantification. RNA retrotranscription to cDNA was carried out using 1 μg total

RNA (DNA free), random hexamers and a High Capacity cDNA Archive Kit (Applied Biosystems). For quantitative real-time qPCR, power SYBR GreenPCR master mix (Applied Biosystems) was employed according to the instructions of the manufacturer.

Oligonucleotides were designed with Primer Express software (Applied Biosystems). The specific primers for eGFP were:

Forward: 5′-GTAAACGGCCACAAGTTCAGC-3′, Temperature: 53.9°C

Reverse: 5′-TGGTGCAGATGAACTTCAGGG-3′, Temperature: 54.9°C

The precise amount of RNA (based on optical density readings) and its quality (lack of degradation) were normalized with respect to an endogenous control gene (glyceraldehyde 3-phosphate dehydrogenase). In any case, glyceraldehyde 3-phosphate dehydrogenase expression was employed as the endogenous gene control but only as a comparison for testing that correct RNA quantities were being employed. Quantitative data were calculated as the number of DNA or RNA copies on a regression curve employing the plasmid containing the eGFP gene. The copy number of eGFP DNA and RNA was always performed on standard curves of the same gene. Data graphical plotting was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, Calif., USA).

Results

Surgical Technique

Premedication was performed with an intramuscular injection of ketamine chloride. After injecting propofol, the animals were intubated and ventilated with a mixture of nitrous oxide, oxygen and sevoflurane. One catheter was placed in the external jugular vein for infusion and blood sampling, and another catheter was placed in the femoral artery for blood pressure monitoring. A complete midline laparotomy was carried out, exposing all the abdominal organs (fig. 1a). The general dissection and exposure of the structures were similar in all cases (fig. 1b). Cannulation differed depending on the injection models. By mobilizing the intestinal package towards the left, the extrapancreatic portal vein was exposed and encircled with one or two vessel loops, depending on the cannulation model involved. The hepatic artery was identified in the hepatic ileum and was referenced with a silk ligature. The inferior vena cava above the renal veins was dissected and referenced with two vessel loops to create a closed segment in the vena cava for cannulation. To encircle the suprahepatic vena cava and the hepatic veins with a vessel loop, liver mobilization by sectioning the retroperitoneal ligaments was required. Likewise, to encircle the suprahepatic vena cava and the hepatic veins with a vessel loop, liver mobilization by sectioning the retroperitoneal ligaments was again necessary (fig. 1c).

This surgical technique allows us to establish three models of perfusion in total hepatic vascular occlusion (fig. 2). In model 1, cannulation is performed through the portal vein and vena cava simultaneously, in model 2 only perfusion through the vena cava is performed, and in model 3 only the portal vein is perfused.

In model 1, and after identifying and referencing all the vascular structures, we proceed to clamp the veins with vascular tourniquets and the hepatic artery with a bulldog clamp. The sequence is as follows: first hepatic artery, then portal vein, and finally infrahepatic vena cava, to interrupt hepatic inflow. The suprahepatic vena cava is clamped last, to obtain total hepatic vascular exclusion. Two longitudinal incisions are made on the anterior surface of the portal vein and the infrahepatic vena cava in the gap between both vessel loops, and the perfusion cannulas are then inserted in each vein.

In model 2, only the infrahepatic vena cava is cannulated and perfused. The initial steps for securing total liver vascular exclusion are similar to those in model 1. The portal vein is clamped, and only a longitudinal incision is made on the anterior surface of the cava vein to insert the perfusion cannula.

Eur Surg Res 2013;51:47–57
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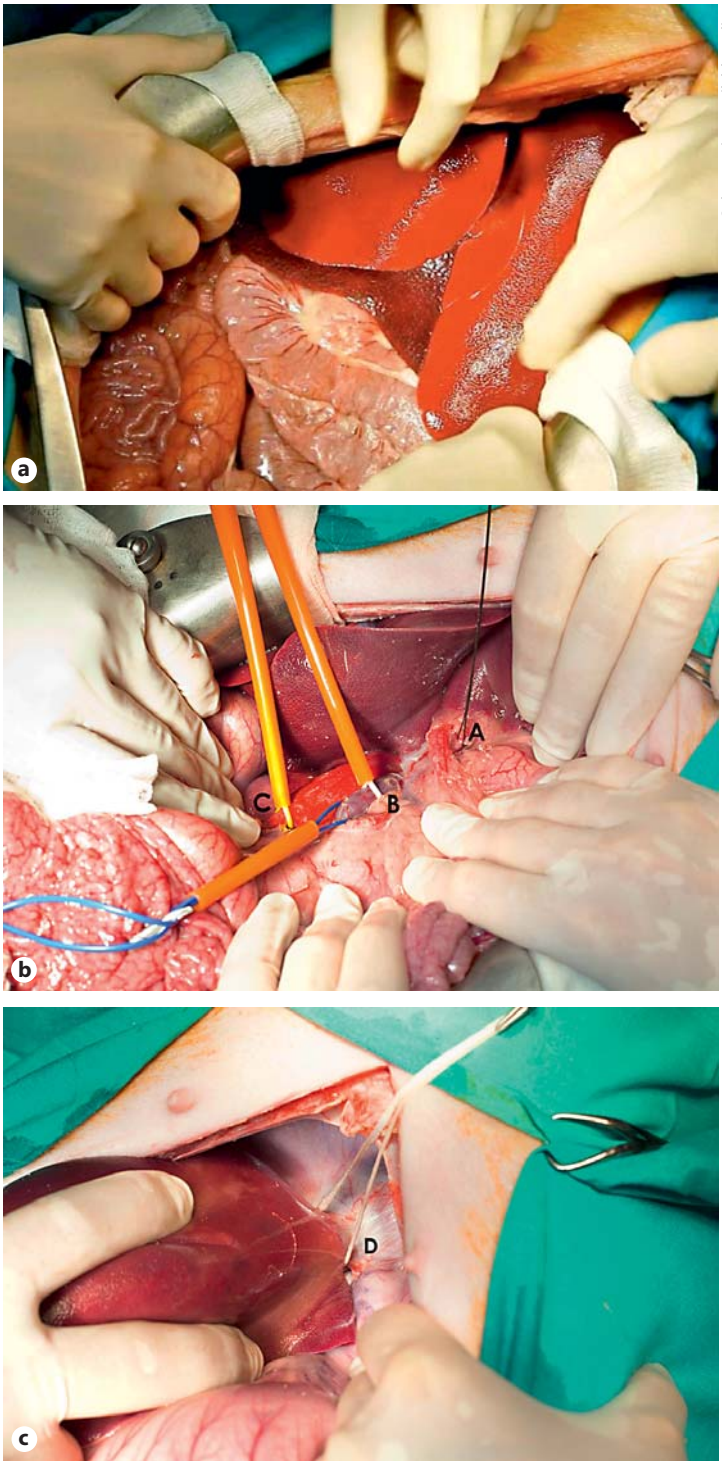

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Fig. 1. Surgical steps. **a** Complete midline laparotomy, exposing all abdominal organs. **b** All the structures are referenced with vessel loops to create a closed segment in the vena cava for cannulation of the vessel. A = Hepatic artery; B = portal vein; C = infrahepatic inferior cava vein. **c** Mobilization of the liver by sectioning the retroperitoneal ligaments. D = Suprahepatic inferior cava vein.

In model 3, the process is the same as in model 2, but with clamping of the vena cava and perfusion through the portal vein.

After solution perfusion, the liver is kept under total vascular exclusion for 5 min to allow gene penetration of the cell nuclei. During this time, the incisions of the veins are sutured with interrupted Biosyn 5/0 (vena cava) or Prolene 6/0 (portal vein), starting with the portal vein.

Eur Surg Res 2013;51:47–57
DOI: 10.1159/000351339

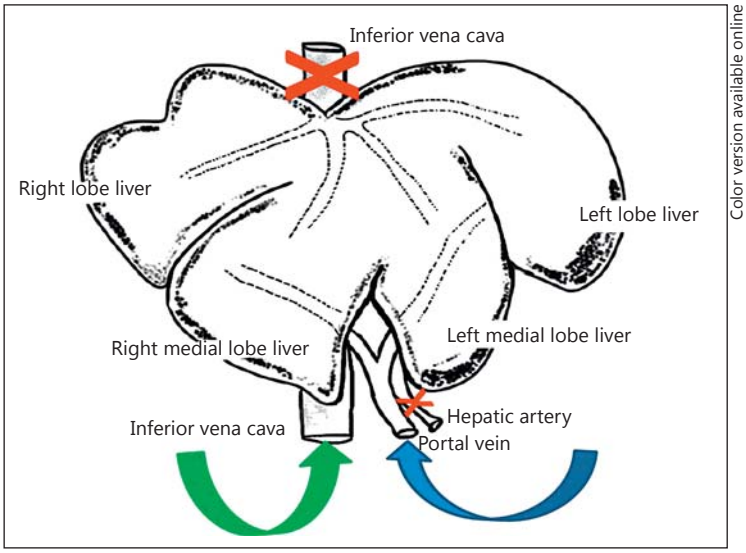

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Fig. 2. Watertight liver and injection model (anterograde, retrograde, simultaneous). Depending on the injection model involved, one of the veins is sealed.

Table 1. Surgery time

Surgical step	Time employed, min (± SD)
Preparation	28.7±8.9
Ischemia	7.8±1.9
Incubation	4.6±0.7
Closure	12.7±3.7
Complete intervention	49.4±11.8

Preparation time = Time from start to injection; ischemia time = total time of hepatic vascular exclusion; incubation time = time of permanence of plasmid in sealed liver; closure time = time from revascularization to the end of the operation; total intervention time = sum of the times of all the surgical steps.

Progressive declamping is carried out in the reverse sequence, first allowing liver outflow and finally inflow through the portal and cava veins.

Hemostasis is checked and the abdominal wall is closed with Dexon 2. The animal is then woken up and sacrificed after 24 h in order to carry out the molecular analysis of the liver lobe samples. This model requires a short space of time as shown in detail in table 1.

Hemodynamic Parameters

Hemodynamic parameters were continuously monitored (table 2) to determine the condition of the animal and ensure the safety of the procedure. Results show a blood pressure decline during liver isolation due to block of venous return, but this proved reversible, and the pressure recovered within a few minutes after revascularization. No important ECG changes were observed, and oxygen saturation was permanently maintained over 98%.

Molecular Analysis

We used model 1 to evaluate the general efficiency of this procedure for gene transfer. In these experiments, 400 ml of DNA solution were injected and two different flow rates (20 and 60 ml/s) were tested. Gene delivery and transcription were studied in each liver lobe sample.

Eur Surg Res 2013;51:47–57
DOI: 10.1159/000351339

Table 2. Hemodynamic parameters during surgery

Surgical step	Hemodynamic parameters				
	systolic pressure, mm Hg	diastolic pressure, mm Hg	heart rate, bpm	$Sato_2$, %	ECG
Basal	92.8±10.0	63.2±9.9	58–60	>98	no change
Vascular exclusion					
Preinjection	46.9±10.0	37.0±6.2	85–88	>98	no change
Injection	59.7±5.6	43.8±4.3	80–84		
Revascularization					
0 min	40.7±5.0	31.3±4.0	89–95		
1 min	77.7±15.9	53.3±10.3	75–80		
2 min	83.8±12.6	57.0±6.3	74–78	>98	no change
3 min	82.5±20.9	60.2±22.3	72–75		
5 min	85.7±7.1	56.0±4.0	70–74		
10 min	93.8±5.5	59.2±2.0	60–63		

Hemodynamic parameters were monitored during the entire intervention. These parameters do not depend on injection conditions but on hepatic vascular exclusion and show low dispersion. Liver isolation lasted no more than 5 min, followed by injection with plasmid solution, which was incubated during 5 min or less. The pigs were subsequently revascularized. Complete recovery occurred in under 5 min.

Table 3. eGFP gene delivery index

Liver injection flow rate	Gene delivery index, eGFP DNA copies/100 pg total DNA	
	20 ml/s	60 ml/s
Right lateral	11.2±1.9	0.6±0.5
Right medial	9.9±0.6	0.7±0.2
Left medial	31.0±20.6$^{\#,\ddagger}$	1.1±0.2*
Left lateral	10.7±0.3	0.7±0.1

Pigs were injected with p3c-eGFP plasmid solution and 24 h later were sacrificed and liver tissue samples were collected from different lobes. Samples were homogenized and DNA was purified. Then, qPCR was carried out to quantify eGFP DNA. Data expressed as eGFP DNA in 100 pg of total DNA. Statistical significance by two-way analysis of variance (ANOVA) analysis and Bonferroni posttest was found in groups marked with * $p < 0.05$ compared with 20 ml/s flow rate group, in groups marked with $^{\#}$ $p < 0.05$ compared with right medial (20 ml/s) and in groups marked with ‡ $p < 0.05$ compared with left lateral in 20 ml/s rate.

For gene delivery, two tissue samples (approx. 100 mg weight) were collected from the distal and proximal areas of each liver lobe and were homogenized. DNA was isolated and purified. qPCR indicated that gene delivery was 10–30 times higher with the 20 ml/s flow rate (9.9–31.0 eGFP DNA copies/100 pg total DNA) than with the 60 ml/s flow rate (0.6–1.1 eGFP DNA copies/100 pg total DNA) (table 3). These data indicate that a larger amount of plasmid was captured in tissue when injection was performed at 20 ml/s than at 60 ml/s and this could be due to the differential elasticity of the liver respect to the volume of hydrodynamic injection. We believe that higher flow rates could expand the vessels and allow the anadromous sense of circulation of the blood escaping from the liver. Gene delivery was higher in the left medial lobe with both flow rates.

Table 4. eGFP gene transcription index

Liver injection flow rate	Gene transcription index, eGFP RNA copies/100 ng total RNA	
	20 ml/s	60 ml/s
Right lateral	15,701.8±5,214.7	12,014.6±6,490.6
Right medial	16,619.7±4,676.9	24,358.4±0.0
Left medial	21,475.8±14,868.0	24,000.3±17,734.8
Left lateral	17,556.6±9,926.0	36,371.6±0.0

Pigs were injected with p3c-eGFP plasmid solution and sacrificed 24 h later; liver tissue samples were collected from different lobes. Samples were homogenized and RNA was purified. Then, qPCR was carried out to quantify eGFP RNA. Data expressed in eGFP RNA copies in 100 ng of total RNA. No statistically significant differences were observed between groups by two-way ANOVA and Bonferroni posttest.

For gene transcription analysis, two tissue samples (proximal and distal) were collected from every single lobe and RNA was extracted and purified. RT-PCR was performed to reversely transcribe RNA into cDNA, and qPCR was carried out. The results (table 4) showed no relevant differences in gene expression between the two flow rates: 20 ml/s (15,701.8–21,475.8 eGFP RNA copies/100 ng total RNA) and 60 ml/s (12,014.6–50,411.1 eGFP RNA copies/100 ng total RNA), respectively. These data demonstrate that the 60 ml/s flow rate affords more efficient gene delivery, because genes are expressed as in the 20 ml/s flow rate but with fewer gene DNA copies. Injection at 60 ml/s showed a higher intrinsic efficacy as lower delivery index (table 3) achieved the same copy number of eGFP RNA than 20 ml/s flow rate. These results suggest that the availability for transcription (nuclear localization) of the DNA delivered at 60 ml/s was higher than at 20 ml/s. Further studies to unravel the cellular distribution of exogenous injected DNA are needed. Despite this fact, we prefer the milder 20 ml/s rate instead of the more efficient 60 ml/s flow rate, because the latter is too aggressive for liver tissue and causes limiting liver capsule distension with no benefit in terms of gene expression.

Liver Toxicity

The liver damage induced by surgically mediated gene transfer was evaluated by measuring plasma levels of ALT and AST. The results (table 5) indicate that the procedure mediates limited and transient liver damage. Accordingly, in both cases small increases in AST were observed 1 h after injection, with a return to near normal levels 1 day after in the operation with the 20 ml/s flow rate. The levels reached (<150 U/l) were not relevant in any case. The ALT concentrations remained unaltered.

Discussion

The liver is the main producer of biological molecules in the body. This makes it an ideal target for gene therapy [2, 3] because it contains the mechanisms needed for physiological molecule production and distribution through the bloodstream. Given these characteristics of the organ, it is necessary to develop a systematic strategy to secure efficient gene delivery, with a view to subsequent transfer to the setting of routine clinical practice.

Accordingly, several strategies have been developed to extend such therapy to human use.

Eur Surg Res 2013;51:47–57
DOI: 10.1159/000351339

Table 5. Aspartate and alanine aminotransferase liver enzymes levels

Enzyme	Sampling time, h	Injection flow rate		Normal values, IU
		20 ml/s	60 ml/s	
GOT/AST, IU	0	38	55	15–55
	1	74	107	
	24	36	151	
GPT/ALT, IU	0	29	34	21–46
	1	34	38	
	24	31	57	

The liver was excluded from vascularization and was injected with p3c-eGFP plasmid solution at two different flow rates (20 and 60 ml/s). Blood samples were collected before injection and again 1 and 24 h after injection. The main liver enzymes were determined.

Firstly, nonviral hepatic gene transfer was carried out in mice. Gene delivery was achieved by injecting large volumes of saline solution containing a tracer gene through the caudal vein at a fast flow rate (hydrodynamic injection). This injection was anterograde. Mice produced the protein encoded by the injected gene (luciferase) for 150 days, and gene expression was seen to decline throughout this period [7]. Long-term (6 months) subtherapeutic expression of human α_1-antitrypsin protein was achieved by Zhang et al. [8]. Subsequently, our group likewise obtained expression of this protein at therapeutic concentrations for 6 months too [9]. In a further study [19], intravital microscopy was used to show that blood flow inversion occurs after injection, with the formation of endocytic vesicles. These results indicated that the pressure exerted by injection and venous return caused the augmentation of intrahepatic pressure, which favored gene solution penetration of the hepatocytes through the cell membrane.

Once expression was achieved in small laboratory animals, the next step consisted of transferring the model to larger animals more similar to humans, such as pigs. Different approaches were used to secure a closed space within the liver in vivo, in order to allow a pressure rise equivalent to that observed in smaller animals. Khorsandi et al. [10] performed balloon-catheter-mediated injection without outflow block, but the resulting gene delivery was not high enough to exert a therapeutic function, thus indicating that the blocking of losses was required. These results were confirmed by subsequent studies [16, 20, 21] in which outflow was blocked from the portal vein in order to pressurize the liver. In the literature, only individual liver segments have been subjected to vascularization block, and gene expression was circumscribed to the injected segment. It would be interesting to achieve efficient gene transfer to the whole liver, in order to produce systemic levels of the protein. A strategy in which the whole liver was watertight in vivo was needed, in which gene solution injection increases the intrahepatic pressure and the target genes effectively reach the hepatocyte nuclei. In the present paper we describe a surgical technique in which liver vascularization is blocked (i.e. the organ is made watertight) for a brief interval of time (<10 min) through vascular ligation in vivo. This procedure does not pose a risk for the animals, as evidenced by other authors [22, 23] who studied liver ischemia for longer periods of time. The technique is invasive (laparotomy) but also easy and rapid (<1 h), and proves harmless for the pigs. Blood pressure drops when the circulation is blocked, but this is followed by quick recovery (in 3 min) when the liver is revascularized. Sawyer et al. [24] described QRS segment prolongation after injecting 1–8% of weight volume at 100 ml/s flow rate in rats. In

our experiments, injecting a volume corresponding to approximately 100% of liver weight (2–3% of body weight) at flow rates of 20 and 60 ml/s, respectively, produced no ECG alterations, though the most aggressive conditions caused limiting extension of the liver capsule (close to rupture point). We evaluated liver toxicity by measuring liver enzyme production with the data showing only limited and transient (24 h) changes. Gene delivery was assessed in two individuals, and the results showed eGFP gene expression in each liver lobe. In sum, we have standardized a reproducible technique for safe and efficient hepatic transfer that may allow us to apply cell and/or viral or nonviral gene therapy techniques. We have developed a procedure of potential clinical use and several local and systemic applications transferable to other organs. Further studies are required to evaluate the optimum perfusion conditions for maximally efficient target gene expression. In our hands, the results obtained showed similar levels of eGFP transcription despite the low delivery achieved at a 60-ml/s flow rate. We consider the perfusion at 20 ml/s to be the optimum as RNA copies of the exogenous gene were similar to those of 60 ml/s but being a more secure model for the animal. Further studies employing mammalian genes would be needed to be able to evaluate the response obtained with clinical potentially applicable therapeutic products.

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Disclosure Statement

The present work contains no conflicts of interest, nor any publication material which would violate any copyright or other personal or proprietary rights of any person or entity.

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