A remarkable permeability of canalicular tight junctions might facilitate retrograde, non-viral gene delivery to the liver via the bile duct

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Research Article

Virtually all research in liver gene therapy involves the intravenous administration of DNA vectors. This, in principle, is an easy approach. However, the intravascular use of immunogenic viruses carries considerable risks, with severe and sometimes fatal reactions in both experimental animals and man. With non-viral systems, achieving high levels of safety, liver specificity, and efficiency after intravenous administration is a huge challenge. The delivery of genes by the bile duct represents an alternative and potentially more favourable route, but has rarely been evaluated.

Effective gene delivery via the bile duct requires retrograde flow of DNA vectors into the microscopic periphery of the biliary tree. This in turn requires displacement of the column of bile upstream of the point of delivery. It is important also to note that the canalicular tight junctions might be expected to restrict DNA uptake by hepatocytes to the canalicular membrane of the hepatocyte, rather than the potentially more favourable basolateral surface. However, on the positive side, the canaliculi are of sufficient size (diameter approximately 1 μm) to permit access of DNA vectors into the hepatic lobule.

The earliest study in this area used large volumes (about 40 ml/kg) of hypertonic “naked” DNA solutions, mainly in mice, and reported a scattering of positive hepatocytes. Obstruction of the hepatic venous outflow was required for optimal gene delivery. A more recent study with naked DNA obtained transfection of about 10% of hepatocytes in mice, but vascular occlusion for three minutes was required. Large (500–800 nm) multilamellar liposomes and haemagglutinating virus of Japan (HVJ) liposomes have been evaluated, but resulted in essentially no gene delivery to hepatocytes.

We wished to establish the accessibility of DNA vectors to the hepatic lobule via the bile duct, and then to evaluate gene delivery using techniques potentially applicable in the clinic. In particular, we restricted our experiments to the use of volumes of DNA solution (up to 10 ml/kg) which did not pose a cardiovascular risk. We also avoided hypertonic solutions, and did not use vascular clamping. We evaluated both normal and acutely damaged livers, and as damaged livers are likely to have increased permeability of canalicular tight junctions, in this regard it is worth noting that, in many clinical situations where gene therapy is being considered, the liver is damaged by metabolic or inflammatory processes.

We have been developing synthetic peptides as non-viral gene delivery systems and have recently described an all peptide gene delivery system highly efficient (>95%) for postmitotic corneal endothelial cells. This consists of a (Lys)₅-containing peptide for binding and condensing DNA, and for translocation of plasmid DNA into the nucleus, and an acid dependent fusogenic peptide for endocytic escape. The capacity to transfect non-dividing cells is a crucial characteristic for any DNA vector system aimed at hepatocytes in normal liver.

MATERIALS AND METHODS

Rats

Inbred LEW rats (Harlan UK, Oxon, UK) weighing 180–240 g were used. All procedures were approved by our institution’s ethics committee.

Surgical technique

Surgical techniques were performed under isoflurane general anaesthesia, using a Zeiss operating microscope for operator and assistant. A 975 syringe pump (Harvard Apparatus, Kent, UK) was used for the perfusions, with a 33 gauge needle for insertion into the bile duct.

A 4/0 silk tie was placed around the bile duct just upstream of the branch from lobes 2 and 3 (which comprise the right

Abbreviations: EGTA, ethylene glycol-bis (β aminoethyl ether) – N, N', N', tetra acetic acid; PFC, perfluorocarbon; RLU, relative light unit.
lateral lobe) (fig 1, tie A). A second tie (fig 1, tie B) was placed about 1 cm downstream, and a loose tie in between. The 33 gauge needle was inserted between the ties, and the loose ligature tightened to prevent backflow. Sometimes the branch from lobes 4 and 5 enters the isolated segment of bile duct, and must be tied separately. The DNA solution (usually 1 ml per 100 g of body weight) was delivered at a standard rate of 0.54 ml/min. The needle was then withdrawn and the middle ligature tightened to prevent leakage. The ties were left in place for two hours.

Induction of liver damage
Rats were given 70 mg per 100 g body weight of D-galactosamine (Sigma-Aldrich, Poole, UK) intraperitoneally. Thereafter, drinking water was supplemented with 5% glucose. 21 Induction of liver damage
One day after gene delivery, the rats were exsanguinated, and 10 ml of ice cold 0.15 M NaCl was infused into the portal vein to remove blood. 24 Lobes 2 and 3, and control lobe 1 (fig 1) were individually removed, small samples taken for histopathology and frozen sections, and the remainder frozen for luciferase assays.

Luciferase reporter gene expression in individual lobes
The liver lobes were thawed, weighed, minced, and 2 ml of lysis buffer (Promega, WI, USA) was added per gram of tissue. The tissue was manually homogenised with a ground glass homogeniser, centrifuged at 16 000 g for 40 minutes at 4°C, and the supernatant stored in aliquots at −80°C. A 20 μl sample of the supernatant was assayed by adding 100 μl of luciferase assay substrate (Promega) and measuring light emission for 10 seconds (Anthos Lucy 1 Luminometer, Labtech International, Uckfield, UK).
The concentration of protein was determined with the Bradford protein assay reagent (Bio-Rad, CA, USA), and luciferase enzyme activity expressed as relative light units (RLU) per milligramme of protein.

β-galactosidase gene expression in frozen sections
The pCMVβ plasmid was used for histochemical localisation of gene expression on frozen sections. 13, 14

Delivery of 10 nm gold particles
A solution of 10 nm gold particles (BBI International, Cardiff, UK) at 1.4×1012 ml in 0.1% bovine serum albumin (BSA) in 5% dextrose/Tris was administered using our standard technique. At the end of the two hour duct ligation, the rat was exsanguinated, and the liver perfused via the portal vein with 20 ml of saline at 5 ml/min. For light microscopy, cubes of liver were snap frozen in liquid nitrogen. Frozen sections were fixed in 1% glutaraldehyde in saline at room temperature for 10 minutes, washed in pure water, and the gold particles revealed by silver enhancement.

For electron microscopy, immediately after the saline perfusion, the liver was perfused via the portal vein with 20 ml of 2.5% glutaraldehyde in phosphate buffer at 5 ml/min. Cubes of liver of 1–2 mm sides were placed in 2% glutaraldehyde, postfixed in osmium tetroxide, dehydrated in ethanol, and resin impregnated. Sections of 80–120 nm were cut and stained with uranyl acetate and lead citrate.

In vitro toxicity assay
The HUH-7 human hepatocyte line (Dr David Crabb, Indiana University Medical Centre, USA) was seeded into a 24 well plate at 5×104 cells/well in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Paisley, UK) containing 10% fetal calf serum, 2 mM glutamine, and 1 mM non-essential amino acids, and cultured overnight at 37°C with 95% air/5% CO2.

Three compounds were evaluated for toxicity: ethylene glycol-bis (β aminoethyl ether) – N, N′, N″, N‴, tetra acetic acid (EGTA) (Sigma-Aldrich), sodium caprate (Sigma-Aldrich), and perfluorocarbon (PFC) compound FC-75 (Acros Organics, NJ, USA). These were diluted in phosphate buffered saline (PBS), and 0.5 ml was added to the wells in triplicate. The cells were incubated at 37°C for two hours, after which the wells were drained, 1 ml of culture medium...
added, and cultured overnight. At 24 hours MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide, Sigma-Aldrich) was added to each well to a final concentration of 0.5 mg/ml, and the reaction product measured as recommended by the manufacturers.

**Size of peptide/DNA nanoparticles**
Dynamic light scattering was performed on a Zetasizer 3000HS (Malvern Instruments, Malvern, UK), which provided the Z-average (Zav) diameters and the polydispersity index.

**Statistical analysis**
The Mann-Whitney non-parametric test was used, and results considered significantly different if \( p < 0.05 \) in two tailed tests.

**RESULTS**

The slow delivery of the DNA solution (2 ml over about four minutes in a 200 g rat) resulted in only mild swelling of lobes 2 and 3, but interestingly this resulted in pallor for three or four minutes, beginning in the second half of the infusion. There was no effect on the other liver lobes. This mild swelling disappeared completely after about 10 minutes, indicating that the injected fluid had been dispersed.

**Access to liver lobule via retrograde infusion into the bile duct**
A solution of 10 nm gold particles was delivered via the bile duct to the right lateral lobe, and the extent of penetration evaluated two hours later. The gold particles clearly penetrated about one third of the way into the liver lobule from the portal triads (fig 2).

**Gene delivery using (Lys)\(_{16}\)-molossin/DNA/fusogenic peptide nanoparticles**
In the case of normal rats, there was essentially no gene delivery in three of six rats tested \((<10^3 \text{ RLU/mg})\), and relatively low in the other three rats (fig 3). However, gene delivery was much higher four days \((p<0.05)\) and seven days \((p = 0.01)\) after D-galactosamine. Gene delivery nine days after D-galactosamine was similar to normal rats. Thus gene delivery using peptide/DNA complexes was more effective in the early stages of liver repair.

**A comparison of naked DNA with peptide/DNA nanoparticles**
Naked DNA at 100 \( \mu \text{g/ml} \) in dextrose/Tris was, surprisingly, more effective than polylysine-molossin/DNA/fusogenic peptide particles in normal rats (fig 4A) \((p<0.05)\) and as effective as the peptide/DNA particles in rats with damaged livers (fig 4B). Naked DNA was equally effective in normal and damaged livers.

**Influence of infusion parameters**
Doubling the volume of DNA solution to 2 ml/100 g body weight, at the standard rate of delivery (0.54 ml/minute), did
not improve gene delivery (fig 4A and B). Reducing the DNA concentration from 100 \( \mu \text{g/ml} \) to 20 \( \mu \text{g/ml} \) in a group of rats with damaged livers (fig 4B) did not cause significant reduction in gene delivery. Delivery of naked DNA at 1 ml/100 g body weight at double (1.1 ml/min) or half (0.28 ml/min) (n = 4 for each group) the standard rate did not affect gene delivery in normal rats (data not shown).

Attempts to increase the permeability of canalicular tight junctions
Loosening of tight junctions between respiratory epithelial cells with PFCs, sodium caprate, and EGTA, both in primary culture and in vivo in the bronchial tree, promotes exposure of the basolateral surfaces of these cells to adenovirus vectors and substantially augments gene delivery.\(^{15}\) We evaluated these agents in our model.

Preliminary in vitro toxicity studies
Perfluorocarbon was without any toxicity (fig 5A). EGTA gave background readings in the MTT assay because the HUH7 cells detached as sheets, presumably because of the chelation of divalent cations. The cells themselves looked in excellent condition by phase contrast microscopy. Sodium caprate caused immediate lysis of all cells as observed by phase contrast microscopy. We therefore proceeded with in vivo evaluation of PFC and EGTA.

In vivo studies
The size and stability of polylysine-molossin/DNA particles are influenced by salt concentrations.\(^{14, 15}\) At 0.4 M and 0.2 M EGTA, no particles were detected, presumably because of disruption or large scale aggregation. At 0.1 M, 0.05 M, 0.025 M, and 0.0125 M EGTA, dynamic light scattering revealed particle diameters of 430 nm, 450 nm, 340 nm, and 200 nm respectively. The diameter of particles without EGTA was 170 nm. The 0.0125 M concentration, previously shown to be effective on respiratory epithelial cells,\(^{26}\) was therefore evaluated. Two normal rats and two rats at day 7 after D-galactosamine were given peptide/DNA particles in 0.0125 M EGTA, but this did not improve gene delivery (fig 5B).
The PFC is not miscible with water. We therefore administered the PFC at 1 ml/100 g of body weight via the bile duct at 0.54 ml/min, and planned to follow this infusion with peptide/DNA particles. However, both rats in which this was attempted experienced breathing difficulties and died within 1–2 minutes of completing the infusion of PFC. A possible explanation is that the PFC molecules (molecular weight 420 daltons) passed through the canalicular tight junctions and caused cardiovascular malfunction or pulmonary embolism. An identical picture was seen when 1 ml of PFC was administered intravenously via the tail vein.

Accessibility to the space of Disse
A possible reason for the improved gene delivery by peptide/DNA particles in damaged livers (fig 3) is loss of integrity of canalicular tight junctions.9 Given that the 10 nm gold particles penetrated into the peripheral one third of the hepatic lobule (fig 2), we performed electron microscopy studies to locate the particles at the ultrastructural level. Surprisingly, the gold particles were present not only in hepatocytes, but also (and more abundantly) in sinusoidal endothelial cells and Kupffer cells (fig 6), clearly indicating ready access to the space of Disse. As a point of reference, the IgG molecule (about 160 000 daltons) is about 8 nm in length.27

To clarify the kinetics of this process, and to further confirm canalicular tight junction permeability, luciferase enzyme (molecular weight 60 750 daltons) (Promega, Southampton, UK) was delivered to the bile duct using our standard protocol, or injected directly into the portal vein. Blood was collected and the RLUs were measured per 20 μl of serum (fig 7). Remarkably, there was no difference between direct injection into the portal vein, and infusion into the bile duct. Blood levels five minutes after injection were approximately 1–2 mg/ml, reflecting an approximate 1 in 15 dilution of the injected 1 ml of luciferase solution.

Localisation of gene expression
At the organ level, luciferase activity was restricted to lobes 2 and 3, with never any reading at all in lobe 1. Given that the sensitivity of the luminometer is about 0.001 RLUs, and average readings were about 10 RLUs, gene delivery outside the targeted lobes was <0.01%.

At the cellular level, the CMVβ plasmid was delivered as naked DNA to four normal rats and four rats treated with D-galactosamine seven days before gene delivery, and evaluated
on frozen sections. There was a scattering of positive cells in lobe 2 of normal (fig 8B) and damaged (fig 8D) livers, with never any positive cells in the control lobe 1 (fig 8A and C) or lobe 2 of four normal rats treated with the control pGL3 plasmid (fig 8E). Under high power (fig 8F) it was clear that many of the positive cells were hepatocytes. There was no gene delivery to biliary epithelial cells.

**Histopathology**

In the normal rats, control lobe 1 and perfused lobes 2 and 3 were histologically normal at the time of harvest. There is substantial hepatocyte necrosis and inflammatory cell infiltration after the administration of D-galactosamine. There was no obvious difference between lobes 2 and 3 as compared with control lobe 1 on the day of harvest in D-galactosamine treated rats.

**DISCUSSION**

Tight junctions at the periphery of the biliary tree (the canaliculi and cholangioles) are the major physical barrier to the diffusion of molecules from bile via the space of Disse into the sinusoids. Water flows freely across these tight junctions into the biliary tree as a consequence of osmotic forces generated by the secretory functions of the canalicular membrane. It would therefore be anticipated that water will flow freely in the opposite direction, from the biliary tree into the space of Disse, under conditions of mildly increased
hydrostatic pressure generated by the infusion of DNA solution into the bile duct. This probably explains why the positive pressure generated by the infusion was rapidly dissipated (about 10 minutes), and that no positive pressure was generated during the two hours of bile duct ligation. This probably also is the basis for the retrograde advance of the infused DNA solution well into the canaliculi of the hepatic lobule.

The remarkable transfer of macromolecules from biliary tree to sinusoids in normal rats, demonstrated by the simple device of retrograde infusion into the biliary tree, was entirely unexpected. A protein of about 60 kDa flowed into the blood as freely via the bile duct as via an intravenous injection. Gold particles of 10 nm, equivalent to molecules of many hundreds of thousand daltons, were found in abundance in sinusoidal endothelial cells and Kupffer cells of the right lateral lobe, two hours after bile duct delivery. This must reflect permeability of canalicular and/or cholangiole tight junctions. If access to sinusoids were exclusively via cholangioles, one might expect the gold particles to be restricted to the immediate vicinity of the triads, which was not the case. In any case, the kinetics and other details of this phenomenon are currently being further evaluated.

For gene therapy, our data show that the simple retrograde infusion of naked DNA via the bile duct can give rise to a scattering of positive hepatocytes in both normal and acutely damaged livers. There is no advantage, and in the case of normal livers there is a disadvantage, in the use of sophisticated peptide/DNA delivery systems. The protocol does not require large volumes of DNA solution, hypertonic solutions, vascular occlusions, nor any forceful or rapid delivery of the DNA. Although it is a mild procedure, longer solutions, vascular occlusions, nor any forceful or rapid delivery of the DNA. Although it is a mild procedure, longer

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