Improved Hepatocyte Engraftment After Portal Vein Occlusion in LDL Receptor-Deficient WHHL Rabbits and Lentiviral-Mediated Phenotypic Correction In Vitro

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Innovative cell-based therapies are considered as alternatives to liver transplantation. Recent progress in lentivirus-mediated hepatocyte transduction has renewed interest in cell therapy for the treatment of inherited liver diseases. However, hepatocyte transplantation is still hampered by inefficient hepatocyte engraftment. We previously showed that partial portal vein embolization (PVE) improved hepatocyte engraftment in a nonhuman primate model. We developed here an ex vivo approach based on PVE and lentiviral-mediated transduction of hepatocytes from normal (New Zealand White, NZW) and Watanabe heritable hyperlipidemic (WHHL) rabbits: the large animal model of familial hypercholesterolemia type IIa (FH). FH is a life-threatening human inherited autosomal disease caused by a mutation in the low-density lipoprotein receptor (LDLR) gene, which leads to severe hypercholesterolemia and premature coronary heart disease. Rabbit hepatocytes were isolated from the resected left liver lobe, and the portal branches of the median lobes were embolized with Histoacryl® glue under radiologic guidance. NZW and WHHL hepatocytes were each labeled with Hoechst dye or transduced with lentivirus expressing GFP under the control of a liver-specific promoter (mTTR, a modified murine transthyretin promoter) and were then immediately transplanted back into donor animals. In our conditions, 65–70% of the NZW and WHHL hepatocytes were transduced. Liver repopulation after transplantation with the Hoechst-labeled hepatocytes was $3.5 \pm 2\%$. It was $1.4 \pm 0.6\%$ after transplantation with either the transduced NZW hepatocytes or the transduced WHHL hepatocytes, which was close to that obtained with Hoechst-labeled cells, given the mean transduction efficacy. Transgene expression persisted for at least 8 weeks posttransplantation. Transduction of WHHL hepatocytes with an LDLR-encoding vector resulted in phenotypic correction in vitro as assessed by internalization of fluorescent LDL ligands. In conclusion, our results have applications for the treatment of inherited metabolic liver diseases, such as FH, by transplantation of lentivirally transduced hepatocytes.

Key words: Hepatocyte transplantation; Liver; Rabbit; Lentiviral vector; Portal vein embolization; Familial hypercholesterolemia

INTRODUCTION

Hepatocyte transplantation has emerged as a viable alternative to orthotopic liver transplantation for treating life-threatening inherited metabolic liver diseases.

However, wide use of this approach is limited by the shortage of donor organs and by the quality of the hepatocyte sources (17,18,49). Indeed, the current technologies for isolating adult hepatocytes yield wide ranges of cell viability and quality (7,32). Moreover, most patients receive

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cryopreserved cells, the functionality of which is substantially lower than that of freshly isolated hepatocytes (51). In addition to the difficulties imposed by the poor availability of human hepatocytes, these cells cannot be substantially expanded in tissue culture (55). Finally, despite immunosuppression, engrafted cells are confronted by reactions mediated by the adaptive immune system, such as cell-mediated or humoral rejection (1,54).

Ex vivo gene therapy requires high levels of hepatocyte transduction because a limited number of hepatocytes are isolated from the resected liver lobe and subsequently transplanted. The development of third-generation self-inactivated HIV-1-derived lentiviral vectors, which efficiently transduce mitotically quiescent primary cell types, including adult human hepatocytes, has prompted new strategies in the field of liver gene therapy (23,39,44). Such approaches should circumvent the major hurdles of allogenic hepatocyte transplantation as they involve the transplantation of freshly isolated autologous hepatocytes. We previously described a novel transduction approach, in which isolated hepatocytes are transduced in suspension with a lentiviral vector and immediately transplanted (SLIT) (8). This approach obviates the need for primary culture, which is a major limitation emphasized in a clinical trial of ex vivo gene therapy in familial hypercholesterolemia (FH) patients (27). In addition, a self-inactivated lentiviral vector expressing green fluorescent protein (GFP) from hepatocyte-specific promoter (mTTR, a modified mouse transthyretin promoter) was designed to transduce hepatocytes isolated from a patient suffering from Crigler-Najjar syndrome type 1 (13). This resulted in long-term transgene expression after hepatocyte transplantation into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse livers (8).

However, hepatocyte transplantation efficacy is limited because the transplanted cells become entrapped in portal spaces and are subsequently destroyed by the innate immune system and the remaining transplanted hepatocytes are poorly engrafted in the liver parenchyma (28,54). Indeed, numerous studies in rodent models have shown that significant liver repopulation occurs only if the resident hepatocytes are destroyed or blocked and if the transplanted hepatocytes display a selective advantage over host liver cells (25,26,57). However, since these procedures used in rodents cannot be transferred to human patients, different regenerative strategies are needed to achieve hepatocyte transplantation success in clinical settings. Partial portal vein embolization (PVE) is a frequently used approach in clinical surgery to increase the remnant liver volume, as it results in hypertrophy of the nonoccluded liver segments. Portal vein occlusion may be achieved by either portal vein ligation or PVE. Both techniques have been shown to induce hepatocyte proliferation and liver regeneration in rodents, pigs, and

nonhuman primates (20,34,56). By combining the SLIT and PVE approaches, we demonstrated that PVE improved engraftment of lentivirally transduced hepatocytes in nonhuman primates, resulting in 6% of engrafted cells (15).

Familial hypercholesterolemia (FH) is a life-threatening human inherited autosomal disease caused by a mutation in the low-density lipoprotein receptor (LDLR) gene leading to severe hypercholesterolemia and premature coronary heart disease (29). The current treatment methods for homozygous FH are either inefficient (diet, bile acid binding resins, statins) or laborious and aggressive (LDL apheresis) (21). The Watanabe heritable hyperlipidemic (WHHL) rabbit is the most accurate animal model of FH. Like FH patients, WHHL rabbits spontaneously develop hypercholesterolemia and atherosclerosis due to genetic and functional LDLR deficiencies (10). Reductions of serum cholesterol levels in WHHL rabbits were achieved through an ex vivo gene therapy approach using a Moloney oncoretrovirus-derived vector and by repeated infusions of allogenic hepatocytes concomitant with ischemic reperfusion (4,12). However, these approaches are either inefficient or not readily transposable to humans (27).

In the present work, we investigated the potential of our ex vivo gene therapy approach in New Zealand White (NZW) rabbits and in WHHL rabbits by evaluating (a) PVE tolerance; (b) the efficacy of the SLIT+PVE approach in terms of hepatocyte transduction, engraftment, and long-term transgene expression; and (c) the in vitro correction of the WHHL hepatocyte phenotype by transduction with a lentiviral vector encoding human LDLR.

MATERIALS AND METHODS

Animals

NZW and WHHL male rabbits aged between 17 and 21 weeks and weighing between 2.5 and 3.5 kg were purchased from Harlan (France) and Centre de Production Animale (Olivet, France), respectively. All animals were housed at the animal facilities of Institut Gustave Roussy (Villejuif, France) for NZW rabbits or Ecole Nationale Vétérinaire de Nantes (Nantes, France) for WHHL rabbits. They were maintained in separate cages on a standard diet and kept in 12-h light–dark cycles. All animals received humane care, and experiments were carried out in accordance with the guidelines of the French Ministry of Agriculture.

Study Design

Ten NZW rabbits were used to define the surgical protocol of reversible embolization using Curaspon® (Curamedical, Zwaneburg, The Netherlands) and the lentiviral transduction conditions. Permanent embolization with Histoacryl® was carried out in 15 NZW rabbits and 7 WHHL rabbits. Nine animals (six NZW and three WHHL) were used to set up the definitive procedures. The 13 remaining rabbits

were transplanted either with Hoechst-labeled hepatocytes (six NZW and three WHHL) or with hepatocytes transduced with lentiviral vectors (three NZW and one WHHL).

Left Lobe Resection and Portal Vein Embolization

Briefly, anesthesia was induced using alfaxalone (Alfaxan®, 2–5 mg/kg, IV) after premedication with dexmedetomidine (Dexdomitor®, 50 µg/kg, IM), and anesthetized rabbits were maintained under isoflurane in 100% oxygen. Rabbits received cephalexin (Rilexine®, 30 mg/kg, IV) for prophylactic antibiotherapy before surgery and morphine (1–2 mg/kg, IV), as required for perioperative analgesia. The abdomen was entered through midline laparotomy. A left lobectomy was carried out, and the left lobe, corresponding to approximately 25% of the liver mass, was used for hepatocyte isolation. Permanent PVE was performed by injecting a biological glue (Histoacryl®, 1:1 mixture of cyanoacrylate and lipiodol), frequently used in clinical surgery. An initial portogram using a 3-F (3-French = 1 mm) introducer placed in the inferior or superior mesenteric vein was taken to map the portal branches before embolization. The two portal branches of the median lobe were embolized distally and proximally using a microcatheter (Terumo Progreat®, Guyancourt, France) that was selectively placed under fluoroscopic control. A second portogram was then performed to determine whether embolization was complete and whether the remaining portal branches were patent. The microcatheter was then replaced by a 3-F venous catheter connected to a perfusion chamber (Access Technologies, Skokie, IL), which was placed subcutaneously in the left anterior thoracic region to provide chronic access to the portal vein. Correct placement and function of the catheter was tested intraoperatively by injecting physiological serum. Transdermal fentanyl (12 µg/h patch-Durogesic®) was used as a postoperative analgesic.

Description and Production of Lentiviral Vectors

The self-inactivating lentiviral vector, mTTR.GFP.WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), has been previously described and encodes GFP under the transcriptional control of the mTTR liver-specific promoter composed of the murine transthyretin promoter fused to a synthetic hepatocyte-specific enhancer (41). In the mTTR.LDLR.WPRE vector, the GFP sequence in mTTR.GFP.WPRE was replaced by the human LDLR cDNA.

Vectors were produced by transient transfection into 293T cells as described (47). Vector titers were determined on HeLa cells by real-time qPCR on an ABI Prism 7900 using SYBR green (MesaGreen qPCR MasterMix, Eurogentec) and primers specific for the vector (GAG-F, GGAGCTAGAACGATTTCGCAGTTA; GAG-R, GGTGTAGCTGTCCCAGTA TTTGTC) or for β -actin (47). Vector titers were routinely 5×10^9 TU/ml.

Hepatocyte Isolation

Hepatocytes were isolated from the left liver lobe by a two-step collagenase perfusion (3). Hepatocytes were purified from nonparenchymal cells by sedimentation on 30% Percoll isodensity solution (GE Healthcare). Hepatocyte viability was determined by trypan blue dye exclusion (viability greater than 95%).

Hoechst Labeling

The freshly isolated hepatocytes were pelleted (50×g, 5 min) and either immediately labeled with Hoechst fluorescent dye (for 30 min at 37°C) in a serum-free culture DMEM/Ham's F12 medium containing 50 µg/ml Hoechst 33258 (Sigma-Aldrich).

Lentiviral Transduction

The freshly isolated hepatocytes were pelleted (50×g, 5 min) and transduced with recombinant lentiviruses at a cell density of 1×10^7 cells/ml in the University of Winconsin solution (ViaSpan; DuPont Pharmaceuticals) containing 50 µM vitamin E succinate (Sigma-Aldrich) on ultralow attachment culture plates (Corning) (1×10^8 cells per plate) for 1 h at 37°C without cell agitation.

Hepatocyte Transplantation

Before transplantation, Hoechst-labeled hepatocytes were washed three times in DMEM/Ham's F12 without phenol-red (Invitrogen), and transduced hepatocytes were washed three times in cell plating medium (hormonally defined and serum-containing medium) (3), then two times in DMEM/Ham's F12 without phenol-red (Invitrogen). Cells were suspended in a saline solution at a cell density of 1×10^7 cells/ml. Samples of Hoechst-labeled or transduced and control hepatocytes were plated and cultured at a density of 67,000 cells/cm² in hormonally defined and serum-free medium on collagen-coated plates as described (3). Transduction efficiency was determined by fluorescence-activated cell sorting (FACS) and fluorescence microscopy 5 days after transduction. Transduced or Hoechst-labeled hepatocytes (between 100 and 150 million) were suspended at a density of 1×10^7 cells/ml in normal saline containing heparin (20 U/ml) and infused at a flow rate of 0.5 ml/min. Portal pressure was continuously monitored during hepatocyte infusion.

Assessment of Blood Parameters and Liver Function After Hepatocyte Transplantation

Liver function was assessed by measuring the concentration of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), gamma glutamyl transpeptidase (GGT), alkaline phosphatase, and total bilirubin in blood samples. These tests were performed at the Routine Biochemistry Department of Nantes veterinary school.

Detection of Transplanted Hepatocytes

Short-term biopsies were performed 4 h or 7 days after transplantation of Hoechst-labeled or lentivirally transduced hepatocytes. Long-term biopsies were performed 56 days after transplantation of transduced hepatocytes. Cryostat sections of animals transplanted with Hoechst-labeled hepatocytes were analyzed using an Axiomager A1 microscope (Carl Zeiss) and an EC Plan-Neofluar lens, 20X-NA 0.5. Paraffin-embedded liver sections (5 μ m) were permeabilized with 0.1% Triton X-100. Endogenous peroxidase activity was blocked with 3% H₂O₂ in PBS for 20 min. Sections were incubated with a mouse anti-GFP antibody (dilution 1:100) (Clontech) for 2 h at room temperature and then with sheep anti-mouse immunoglobulin linked to horseradish peroxidase (dilution 1:100) (Amersham) for 1 h. The peroxidase reaction was carried out with 3'-diaminobenzidine (DakoFrance). Twenty sections of each nonembolized liver segment were analyzed at random levels on different biopsies (20 $\times$ magnification). At least 1,200 hepatocytes per biopsy were counted in the different sections.

Vector Copy Number Quantification

High-molecular-weight DNA (200 ng) from harvested organs was isolated using the Qiagen Genomic Tip 20/G kit (Qiagen). Genomic DNA was subjected to amplification by real-time quantitative PCR on an ABI Prism 7300. For these reactions, we used WPRE-F and WPRE-R primers and WPRE-P probe (Eurofins) specific for the WPRE sequence in the lentiviral vector (Table 1). For normalizing the amount of genomic DNA, we used RabB2-F and RabB2-R primers and RabB2-P probe specific for the β -actin gene (Table 1). Amplification conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 10 s, 60°C for 1 min. A standard curve was constructed by using dilutions of lentiviral vector plasmid in genomic DNA extracted from NZW rabbit liver to simulate the presence of 100–0.01 vector copies per genome. Vector copy numbers were calculated by interpolating

Ct(WPRE) and Ct(RabB2) sample values to standard curve values.

Functional Measurement of LDLR Expression by LDL Internalization Assays

WHHL rabbit hepatocytes were transduced with the mTTR.hLDLR.WPRE vector in suspension and cultured as described above. Five days after transduction, hepatocytes were washed to remove dead cells and incubated with 5 μ g/ml of bodipy-LDL (Molecular probes) in the (serum-free) hepatocyte culture medium for 1 h at 37°C. They were then trypsinized and fixed in 2% formaldehyde before FACS analysis. Nontransduced cells served as a negative control. LDL uptake by hepatocytes was assessed by fluorescence microscopy after incubation of the cells with 10 μ g/ml Dil-LDL (Molecular Probes) for 3 h at 37°C, washed with phosphate-buffered saline (PBS), and fixed in 3% formaldehyde.

Western Blot Analysis

Cells were detached using cell scrapers, lysed in radio-immunoprecipitation assay buffer (1% nonylphenoxy-polyethoxyethanol, 0.1% sodium dodecyl sulfate, and 0.5% sodium deoxycholate in 1 $\times$ PBS), and centrifuged to eliminate the cellular debris. Protein extracts were resolved on a NuPAGE 10% Novex Bis-Tris Gel (Invitrogen). The proteins were then transferred to a nitrocellulose membrane. After blocking in 5% skim milk, immunoblots were sequentially incubated with polyclonal goat anti-human LDLR antibody (R&D Systems) or monoclonal mouse anti-actin (Sigma-Aldrich, clone AC-40). The membrane was then washed and incubated for 1 h with appropriate secondary antibodies conjugated to horseradish peroxidase (HPO). Membranes were then washed and developed with an HPO substrate to detect bound antibodies.

Statistical Analysis

A Mann–Whitney *U* test was used to compare quantitative data ($p < 0.05$). Data are represented as mean

Table 1. Primer and Probe Sequences for Vector Copy Number Quantification

Name	Sequence	Concentration (nM)
WPRE-F	CCG TTG TCA GGC AAC GTG	400
WPRE-R	AGC TGA CAG GTG GTG GCA AT	400
WPRE-P	5'-FAM-TGC TGA CGC AAC CCC CAC TGG T -3'-TAMRA	100
RabB2-F	AGC AGA TGT GGA TCA GCA AGC	400
RabB2-R	CCG TTA GGT TTC GTC GAG AGA	400
RabB2-P	5'-FAM-CGC AAG TGC TTC TAG GCG GAC TGT T-3'-BHQ1	250

Figure 1. Effects of left lobectomy and portal vein embolization (PVE) on rabbit liver. Successive schematic anatomy of the liver (left) and portograms (right) are shown: (A) before surgery (double arrows indicate lobe to be resected), (B) immediately after left lobectomy, and (C) after embolization of the median lobe (gray) of the liver. The right portal branch of the liver remained patent. Rabbit liver lobes: ML, median lobe; LLL, left lateral lobe; SRL, right lobe divided into superior right lobe; IRL, inferior right lobe; CL, caudate lobe.

Figure 2. Transduction of rabbit hepatocytes. (A) Dose–response of rabbit hepatocytes to lentiviral transduction. Hepatocytes isolated from New Zealand White (NZW) rabbits were transduced in suspension with increasing amounts of mouse transthyretin–green fluorescent protein–woodchuck hepatitis virus posttranscriptional regulatory element (mTTR–GFP–WPRE) lentiviral vectors. The percentages of GFP-positive and viable cells were determined by fluorescent-activated cell sorting (FACS) at day 5 posttransduction. The data (mean $\pm$ SEM) are representative of three independent experiments. (B) Representative FACS analyses showing mock-transduced Watanabe heritable hyperlipidemic (WHHL) hepatocytes (left) and WHHL hepatocytes transduced at a multiplicity of infection (MOI) of 30 (right). FL2-H and FL1-H represent unspecific fluorescence and specific GFP fluorescence, respectively. (C) Fluorescent microscopy of GFP-expressing hepatocytes in WHHL rabbits. Arrow indicates a GFP-expressing hepatocyte with two nuclei. (D) Morphology of the transduced hepatocytes in WHHL rabbits under phase contrast microscopy. Original magnification: 20 $\times$.

values $\pm$ SEM. Statistical analysis was performed using Graphpad Prism software.

RESULTS

Experimental Protocol

The first series of 10 NZW rabbits was subjected to partial PVE with gelfoam prior to transplantation of Hoechst-labeled hepatocytes. However, we observed very low engraftment efficiency ($<0.5\%$, data not shown). Therefore, we decided to use only permanent PVE using Histoacryl®. We performed portography before and after PVE to document the liver vascular anatomy and afterward to record the embolized liver lobes. Figure 1A depicts the normal liver anatomy and indicates the resected lobe as well as the embolized and nonembolized lobes. Portograms showed that the remnant liver lobes amounting about 30% of the portal territory were patent after the surgical procedures (Fig. 1B, C). This procedure was tolerated by all animals.

Susceptibility of Rabbit Hepatocytes to Lentiviral Vectors

To determine the susceptibility of nonadherent rabbit hepatocytes to lentivirus transduction, we incubated NZW hepatocytes for 1 h with increasing amount of a lentiviral vector encoding GFP under the control of the liver-specific mTTR promoter (mTTR-GFP-WPRE) as previously described (43). The level of transduction reached a plateau of 65–70% at a multiplicity of infection (MOI) of 10 (10 transduction units per cell) (Fig. 2A). However, we observed a 1.5-fold higher expression of GFP at a MOI of 30 as compared with a MOI of 10, as assessed by the mean intensity of fluorescence quantified by FACS (data not shown). The same protocol was therefore performed on WHHL hepatocytes using a MOI of 30, and these cells were transduced as efficiently as normal hepatocytes (Fig. 2B). WHHL hepatocytes expressed high levels of GFP as demonstrated by FACS and fluorescence microscopy analyses (Fig. 2B, C). Transduced hepatocytes with two nuclei were visible, a characteristic feature of hepatocytes. Transduced cells also maintained a regular polygonal shape and contained the round nuclei that typify differentiated hepatocytes (Fig. 2D).

Tolerance of the Procedure and Hepatocyte Transplantation

Hepatocytes were isolated from the left liver lobe of NZW and WHHL rabbits and were immediately transplanted to the remaining liver. In a preliminary study, we observed that rabbits receiving transplantation of greater than 150 million hepatocytes died within a few hours after transplantation. Histological examinations of livers from these animals revealed portal vein thrombosis

(not shown). Consequently, we transplanted between 100 and 150 million hepatocytes in the present experiments. All animals survived. Portal vein pressure moderately increased after the PVE procedure from 9.14 ± 1.21 mmHg before embolization to 11.57 ± 0.97 mmHg after left lobectomy and PVE ($p=0.063$). It returned to normal values before cell transplantation (Fig. 3A). It transiently increased to 14.85 ± 1.21 mmHg at the completion of hepatocyte transplantation ($p=0.0019$) and return to normal values 2 h after transplantation.

We measured serum ASAT and ALAT levels as markers of hepatocyte injury as well as GGT and bilirubin in WHHL rabbits. Serum ASAT levels increased transiently, returning to normal values within 1 month after all procedures were completed (Fig. 3B). Serum ALAT, bilirubin, and GGT levels did not change significantly (Fig. 3B).

To document the fate of transplanted hepatocytes, we performed histological studies of liver tissue samples taken 4 h after cell transplantation was completed in WHHL rabbits. Histoacryl was visible in the lumens of the occluded portal veins (Fig. 3C). Most of transplanted hepatocytes were located in the portal spaces of the nonembolized liver lobes (Fig. 3D). Some islands of transplanted hepatocytes were located within the sinusoidal lumen and were in the process of being integrated (Fig. 3E). Figure 3F shows islands of integrated hepatocytes associated with inflammation.

Engraftment of Transplanted Hepatocytes After PVE

We evaluated short-term engraftment of transplanted hepatocytes. A first series of NZW rabbits was subjected to PVE procedure ($n=6$) and underwent transplantation with $1.3 \times 10^8 \pm 0.2 \times 10^8$ Hoechst-labeled cells. Seven days after transplantation, Hoechst-labeled hepatocytes represented $3.5 \pm 2\%$ of the hepatocytes in the nonembolized liver segments of transplanted rabbits (Fig. 4A, B). No labeled hepatocytes were observed in the embolized segments.

A second series of rabbits (three NZW and one WHHL) were subjected to the PVE procedures and transplanted with $1.2 \times 10^8 \pm 0.3 \times 10^8$ hepatocytes that had been transduced with the mTTR-GFP-WPRE lentiviral vector at a MOI of 30. Seven days after transplantation, GFP-expressing hepatocytes represented $1.3 \pm 0.7\%$ of the hepatocytes in NZW rabbit livers and 1.5% of the hepatocytes in the WHHL rabbit. Figures 4C and D show representative fields of engrafted hepatocytes expressing GFP distributed within the liver parenchyma of the transplanted WHHL rabbit. Small and large cell clusters of transduced hepatocytes were evenly distributed within the parenchyma, suggesting that the transplanted cells had proliferated. Inflammatory cells were visible in some portal spaces in the vicinity of transduced hepatocytes. No GFP-expressing hepatocytes were observed in the embolized segments (not shown). Analysis of biopsies

Figure 3. Tolerance of the surgical procedures and histological analysis of transplanted hepatocytes. (A) Portal pressure values before left lobectomy and PVE (pre-LB/Pre-PVE), after left lobectomy (LB), before cell transplantation (before Cell T), at the end of cell transplantation (End Cell T), and 2 h after the end of cell transplantation (2 h after cell T) in NZW rabbits. (B) Mean values of liver function tests before and after surgical procedures and cell transplantation. D, days; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; GGT, gamma glutamyl transpeptidase; Bilirubin, total bilirubin in WHHL rabbits. (C) Hematoxylin–eosin staining of a representative cryostat section of the embolized lobe showing the presence of Histoacryl® in the lumen of a portal vessel in WHHL rabbits. (D, E, F) Histological analysis of WHHL rabbit liver tissue showing transplanted hepatocytes trapped within the portal tract (D, arrows), located within the sinusoidal lumen (E, arrows), and in the process of being integrated and surrounded by an inflammatory reaction (F, arrows). (E) is a magnification of the area in the square in (D). Original magnification: 10× (C, D), 40× (E), 20× (F).

Figure 4. Engraftment of transplanted transduced hepatocytes. (A, B) Representative images of Hoechst-labeled hepatocytes in NZW liver sections 7 days after transplantation. (C, D) Immunohistochemical staining of representative sections of transduced hepatocytes expressing GFP at day 7 after transplantation in a WHHL rabbit. (E, F) Immunohistochemical staining of representative sections of GFP-positive hepatocytes 56 days after transplantation in a NZW rabbit. Original magnification: 20 $\times$.

collected 56 days after transplantation revealed clusters of GFP-positive hepatocytes (Fig. 4E and F). Engrafted hepatocytes represented 1.8% of the hepatocytes in these biopsies, suggesting that the lentiviral vector had not been silenced.

We also analyzed genomic DNA samples by real-time quantitative PCR to quantify the vector copy number per genome in the livers of transplanted NZW rabbits. We found vector copies to be present in 3.5% and 4% of the diploid genomes in nonembolized liver segments at 7 and 56 days posttransduction, respectively. These results confirm the persistence of the transplanted hepatocytes.

Construction and Functionality of a Lentiviral Vector Encoding LDL Receptors

We generated a third-generation self-inactivating lentiviral vector encoding the human LDL receptor under

the transcriptional control of the liver-specific mTTR promoter. To assess the functionality of this mTTR-hLDLR-WPRE vector and the hLDLR protein, we transduced WHHL hepatocytes with mTTR-hLDLR-WPRE recombinant lentivirus and performed three different analyses. We detected human LDLR protein in transduced WHHL hepatocytes using a Western blot analysis (Fig. 5A). We next used flow cytometry to assess the binding and uptake of fluorescent LDLs (Bodipy-LDL) in lentivirally transduced cells. FACS analysis showed that up to 70% of the transduced hepatocytes had taken up the fluorescent LDL (Fig. 5B). Fluorescence microscopy revealed that transduced hepatocytes were also able to internalize Dil-LDL, another fluorescent ligand of LDL receptor (Fig. 5C). Altogether, these data demonstrate that lentiviral vectors can efficiently transduce WHHL rabbit hepatocytes and can be used to correct their mutant phenotype.

Figure 5. Phenotypic correction of WHHL rabbit hepatocytes. Hepatocytes isolated from WHHL rabbits were transduced with a lentiviral vector encoding human low-density lipoprotein receptor (LDLR). (A) Western blot analysis of cell extracts from transduced (LV) and mock-transduced (NT) hepatocytes using antibodies against actin and human LDLR. Binding and uptake of LDL cholesterol by transduced hepatocytes (LV) was evaluated by FACS using bodipy-LDL (B) and by fluorescent microscopy using Dil-LDL (C). Representative image of fluorescent hepatocytes demonstrating the phenotypic correction of WHHL hepatocytes (C, right). Mock-transduced cells were used as negative control (NT).

DISCUSSION

Basically, ex vivo gene therapy approach requires optimized cell engraftment, efficient gene delivery into target cells, stable transgene expression, and functionality of the transgene's product.

Hepatocyte engraftment in the liver is poor in absence of any liver conditioning. We previously showed in a non-human primate model that PVE is a clinically relevant procedure of inducing regeneration in nonoccluded lobes and that it improves the liver's ability to be repopulated by transplanted hepatocytes (14,34). In an effort to transfer this approach to a large animal model of a metabolic liver disease, we investigated the feasibility and efficacy of our approach in normal rabbits and in the WHHL rabbit model of FH. We evaluated their tolerance to PVE, the effect of PVE on hepatocyte engraftment, the transduction and correction of diseased WHHL hepatocytes by lentiviral vectors, and the in vivo long-term expression of the transgene.

Unexpectedly, we found that the protocol defined in nonhuman primates using transient partial embolization with gelfoam was not transferable to the rabbit. Indeed, this procedure did not allow significant engraftment of transplanted hepatocytes. Although, we did not focus on this aspect, a possible explanation is that the gelfoam was rapidly degraded by the proteases present in the extracellular matrix and that subsequent repermeabilization of the occluded lobes occurred too quickly to allow significant liver regeneration. We then performed permanent PVE on the rabbits, which we also previously described in monkeys (14). The complete surgical procedures were well tolerated by the rabbits, which exhibited only transient elevations in serum ASAT levels. However, in contrast to results in similar-sized monkeys, which tolerated the transplantation of at least 300 million cells, infusion of more than 150 million cells into the NZW and WHHL rabbits resulted in fatal portal thrombosis. Therefore, we had to decrease the number of transplanted hepatocytes.

Within hours after cell transplantation, we observed that the transplanted hepatocytes rapidly translocated from the lumen of the portal space into the liver parenchyma. We also observed inflammatory infiltrates surrounding transplanted cells. These data are in agreement with previous studies in rodents, which showed that within 6 h after cell transplantation, deposition of hepatocytes in liver sinusoids promoted accumulation of inflammatory cells (e.g., Kupffer cells, lymphocytes, and granulocytes), which secreted proinflammatory cytokines (22,33). These inflammatory reactions are directly deleterious to hepatocyte engraftment.

A species-specific barrier to early steps of HIV infection was described in nonhuman mammalian cells. This results in a low infectivity of HIV-1-derived lentiviral vectors in cells from some nonhuman primate species, rodents, and rabbits (6,30,44,50). However, we showed

that this resistance to lentiviral transduction was mostly overcome in rodent hepatocytes by adding vitamin E to the transduction medium (44). Similarly, vitamin E allowed up to 65–70% of NZW and WHHL hepatocytes to be transduced without any apparent toxicity.

Assuming that liver tissue contains $\sim 1 \times 10^8$ cells/g and that rabbit livers weigh ~ 100 g, each liver in our study contained $\sim 1 \times 10^{10}$ hepatocytes. With the expectation that cell engraftment efficiency would be less than 50%, 1.2×10^8 transplanted cells was equivalent to at most 0.60% of the liver in the absence of PVE. Engrafted cells represented $1.3 \pm 0.7\%$ of the hepatocytes in the livers of transplanted animals on posttransplantation day 7. Given the transduction efficiency of 65–70%, this corresponded to a proportion of at least 2% of the liver cells, which was close to that obtained with the Hoechst-labeled hepatocytes. This result shows that PVE significantly increased the proportion of engrafted hepatocytes in rabbits. It was nonetheless lower than that obtained in the macaque model, which might be partly due to the lower number of transplanted cells. Long-term survival of transduced hepatocytes was confirmed by qPCR analysis, which detected the presence of 0.04 copies of integrated vector per diploid genome. According to the level of liver repopulation, these data suggest that the transplanted cells contained a few vector copies per cell (<5). Finally, our preliminary data on long-term GFP expression in a transplanted rabbit suggest that the mTTR promoter is not silenced in vivo. This agrees with previous reports of hepatocyte transplantation and in vivo lentiviral vector delivery in other animal models (9,37,40,42).

In clinical trials of hepatocyte transplantation, allogeneic donor hepatocytes generally represented 5–10% of the recipient liver mass (17). Partial metabolic benefit observed in transplanted patients was variable, and there is no relationship between the number of hepatocytes infused and disease improvement. For example, infusion of 7.5×10^9 hepatocytes resulted in a decrease of 30–50% of bilirubinemia in three children (8–10 years old) with Crigler-Najjar type 1 (2,16,19). In another 8-year-old child with Crigler-Najjar type 1, a 30% decrease in bilirubinemia was achieved after infusion of much less cells (1.4×10^9) (1). An improvement of glucose control in normal diet was observed after transplantation of 2×10^9 cells in a 47-year-old patient with glycogen storage disease type 1 (38). It has been estimated that correction levels between 2% and 5% should benefit to homozygous FH patients. In the first and so far only clinical trial of ex vivo gene therapy, three out of the five FH patients had a long-term 6–20% reduction of LDL cholesterol levels after transplantation of less than 3% of the total liver mass. Since transduced hepatocytes represented 20% of the total number of the transplanted hepatocytes, this suggests that 0.6% of corrected cells could lead to a decrease

of LDL-cholesterol (27). Of note, in the model of WHHL rabbits lower plasma cholesterol levels were detected after a gene correction level of less than 0.01% was obtained by in vivo delivery of lentiviral vectors (31). In our study, transplanted rabbits received hepatocytes at a number equivalent to 3–5% of the remaining liver mass. Our therapeutic lentiviral vector transduced and restored LDLR activity in 65–70% of hepatocytes (i.e., 2–4% of the liver mass). Thus, transplanted hepatocytes expressing LDLR should repopulate WHHL rabbit livers to the same extent as that obtained with transplanted hepatocytes expressing GFP. This suggests that we should observe a decrease in serum cholesterol of the transplanted rabbits. According to our studies in monkeys (14), we can speculate that, compared with rabbits, it will be feasible to safely transplant a higher number of hepatocytes in humans.

Another important criteria to be considered for efficient cell engraftment is the functionality of the hepatocytes before transplantation. Although there is not yet a true consensus on the most appropriate tests to estimate hepatocyte quality, additional criteria other than viability measurements are needed (46). Different rapid assays have been developed to test the competence of cryopreserved and freshly isolated hepatocytes for allogenic transplantation and these could be used in ex vivo gene therapy approaches to predict and improve hepatocyte engraftment (24,52).

A general caveat of the ex vivo approach is that it cannot be repeated. Recent progress in the field of induced-pluripotent stem cells (iPS) generated from somatic cells harvested from patients suggests that, in the near future, these cells could become an additional source of autologous hepatocytes in which deficiencies might be corrected (58). These cells could be then repeatedly infused into livers after reversible PVE. Alternatively, PVE can be envisaged to improve allogenic transplantation of hepatocytes either isolated from a liver donor or generated from different stem cell sources (5,11,36,48,53). Finally, PVE can be also envisioned to improve efficacy of in vivo liver gene therapy approaches using lentiviral or adenoviral vector by priming hepatocyte into cell cycle and/or opening liver fenestrations (35,45).

In conclusion, our approach allows in vitro phenotypic correction, improved hepatocyte engraftment, and sustained transgene expression in a model of FH. Further studies are ongoing in WHHL rabbits to validate the therapeutic potential of this approach in vivo for the treatment of FH.

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