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Efficient ex vivo gene transfer into non-human primate hepatocytes using HIV-1 derived lentiviral vectors

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***Background/Aims*: Lentivirus-mediated ex vivo gene therapy is becoming a promising approach for the treatment of liver metabolic disorders. However, the feasibility of this approach needs to be studied in large animal models. The purpose of this study was to evaluate the efficacy of ex vivo gene transfer into Macaca hepatocytes with two different HIV-1 derived lentiviral vectors.**

***Methods*: A self-inactivating lentivector was constructed to express GFP under the control of the hepatic apolipoprotein A-II promoter. Freshly isolated and thawed hepatocytes were transduced in suspension with lentiviral vectors expressing the GFP gene under the control of a ubiquitous promoter (EF1-α) and the apolipoprotein A-II promoter. Transduced thawed hepatocytes were transplanted into the spleen of newborn mice, and livers analyzed 4 and 12 weeks after transplantation.**

***Results*: We show that lentivectors are efficient in transducing hepatocytes in suspension either freshly isolated or cryopreserved. We also show that thawed and transduced hepatocytes engrafted and participated in liver growth after transplantation into newborn mice and that the apolipoprotein A-II promoter is functional.**

***Conclusions*: Our data show that transplantation of transduced hepatocytes into monkeys should allow to evaluate the fate of transplanted cells and transgene expression in a pre-clinical model of ex vivo gene therapy.**

***Keywords*: Primate; Hepatocytes; Lentiviral vectors; Hepatic promoter**

1. Introduction

Hepatocyte transplantation has become an alternative to orthotopic liver transplantation for the treatment of inherited metabolic disorders. Clinical trials using allogeneic hepatocytes have proven the safety of this approach, and in a small number of cases, it resulted in a transient and partial correction of the disease [1]. Auto-transplantation of ex vivo genetically modified hepatocytes is now considered as an alternative strategy. It would avoid the need for donor liver and immunosuppression problems; indeed grafted cell rejection has been reported after allo-transplantation [2]. Ex vivo gene therapy appears safer than in vivo liver gene transfer as it avoids systemic dissemination of viral particles.

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Virus injection also results in virus sequestration by Kupffer cells, which limits the efficacy of hepatocyte transduction [3]. Only one clinical trial has been performed by auto-transplantation of oncoretrovirally transduced hepatocytes [4] and this approach has been hampered by the low efficiency of hepatocyte transduction. Hepatocytes do not divide more than once in vitro, resulting in only 25–50% of cells being infected [5,6]. Human immunodeficiency virus (HIV)-1-derived vectors efficiently transduce mitotically quiescent primary cell types including hepatocytes [7,8], and lentiviral-mediated transduction of hepatocytes does not require cell division. Moreover, these vectors deliver genes into both adherent and non-adherent hepatocytes making this approach an attractive system [9,10]. However this therapeutic approach requires high transduction efficiency and long-term sustained transgene expression. Vector silencing is a major concern. In rodent livers the CMV promoter is rapidly silenced when on either oncoretroviral-based vectors or lentiviral vectors [11–14], and ubiquitous or tissue-specific promoters other than viral ones need to be evaluated in hepatocytes. Therefore studies in large animal models are needed to assess the strength and long-term activity of candidate enhancer/promoter sequences to drive the expression of a therapeutic transgene. Establishing hepatocyte cryopreservation methods that maintain cell viability and susceptibility of thawed cells to lentiviral vectors may also be important for future clinical developments.

Non-human primates are an appropriate model for cell therapy because the hepatic anatomy and vasculature are very similar to those of humans [15]. We previously developed a protocol of auto-transplantation with in vitro oncoretrovirally transduced hepatocytes in a Macaca model [16]. However, the use of lentiviral vectors to transduce simian hepatocytes has not been described. We explored the potential of simian hepatocytes to establish a lentivirus infection model and we constructed a lentivector containing the GFP gene under the control of the liver specific apolipoprotein A-II (ApoA-II) promoter [17]. We show that Macaca primary hepatocytes, whether freshly isolated or cryopreserved, were efficiently transduced by lentivectors, were able to engraft in the liver of newborn immunodeficient mice and expressed the transgene 12 weeks after transplantation.

2. Experimental procedures

2.1. Hepatocyte isolation, culture and cryopreservation

Monkeys (*Macaca fascicularis*) were housed in the Institut National de la Recherche Agronomique, Jouy-en-Josas, France. Experiments were performed in accordance with the guidelines of the French Ministry of Agriculture. The left lateral lobe was resected and the hepatocytes were isolated by two-step collagenase perfusion from 7 animals [15]. Hepatocytes viability was determined by Trypan blue exclusion. The cells were seeded onto Falcon® Primaria® culture dishes (BD Biosciences) at a density of 67,000 cells/cm^2 and cultured as previously described [16].

Fresh hepatocytes were centrifuged at 50*g* for 5 min and suspended at a density of 5×10^6 cells/ml in the freezing solution: University of Winconsin solution (ViaSpan™, Dupont Pharmaceuticals) containing 12% DMSO, 10% fetal calf serum, 50 μM vitamin E succinate (Sigma). The cryotubes were incubated at −20 °C for 2 h, then at −70 °C for 16 h, and finally stored in liquid nitrogen. The cells were thawed by incubating the cryotubes at 37 °C. Thawed cells were immediately suspended in 10 ml plating medium [16], centrifuged at 50*g* for 5 min, transduced and/or plated onto Biocoat® Collagene I-coated culture plates, which significantly improved the plating efficiency of cryopreserved cells.

2.2. Lentiviral vectors

The vectors express the enhanced GFP gene under the control of the human ApoA-II promoter or the human elongation factor 1-α (EF1-α) promoter [17,18]. To construct the ApoA-II-GFP vector, a *Hin*dIII–*Eco*RI fragment containing the ApoA-II promoter was blunted and ligated into pRRLsincppt-PGK-WPRE [19] digested with *Pml*I–*Eco*RV to excise the PGK promoter. Titers of vectors were determined on HeLa cells and were 2×10^9 HeLa transducing units/ml [9].

2.3. Hepatocyte transduction

Hepatocytes were incubated with lentiviral particles at a density of 10^6 cells/3 ml plating medium. After 4 h at 37 °C, hepatocytes were washed, plated and cultured as described above.

As control for pseudo-transduction, simian hepatocytes were pre-incubated for one hour with 1 μM, 3 μM, or 10 μM 5′-azido thymidine (AZT, GlaxoSmithKline) before transduction at a multiplicity of infection (MOI) of 30. AZT was then added every 24 h to the culture medium. Cells were harvested after 4–5 days of culture with trypsin–EDTA (Invitrogen) and fixed in PBS–paraformaldehyde 1% and GFP expression analyzed by means of flow cytometry (LSR 1, BD Biosciences).

2.4. Immunohistochemistry

Simian hepatocytes, cultured for 3 days on Primaria dishes or on Collagene I-coated 6-well chambers, were fixed for 10 min in 4% paraformaldehyde and permeabilized with PBS–0.1% Triton then saturated with gelatin 1% in PBS for 1 h.

E-cadherin was detected using a monoclonal mouse antibody (1:50) (NCH-38, Dako Corp.); alpha-1-anti-trypsin was detected using a polyclonal, anti-human protein rabbit antibody (1:100). Mouse primary antibodies were detected with Cy3-labeled goat anti-mouse F(ab′)$_2$ fragments (Jackson ImmunoResearch Laboratories); rabbit primary antibodies were revealed with Alexa Fluor® 488 (Molecular Probes). The slides were mounted with a DAPI mounting medium (Vector Technologies).

2.5. Assessment of hepatocyte function

Glycogen storage was assayed by the Periodate-Schiff technique according to McManus. RNA expression was measured by RT-PCR with primer–probe sets (Table 1) specific for human targets which all cross-reacted with simian RNA. Amplifications were performed with TaqPlatinium (Invitrogen).

Cell culture supernatants were tested for albumin and urea concentrations 48, 72 and 96 h after plating. Urea concentrations were measured using an automat (Modular Roche). Albumin concentrations were measured with a kit specific for human protein (Dade Behring). Values were corrected for the blank value of the culture medium.