Hepatic lentiviral gene transfer prevents the long-term onset of hepatic tumours of glycogen storage disease type 1a in mice

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Abstract

Glycogen storage disease type 1a (GSD1a) is a rare disease due to the deficiency in the glucose-6-phosphatase (G6Pase) catalytic subunit (encoded by G6pc), which is essential for endogenous glucose production. Despite strict diet control to maintain blood glucose, patients with GSD1a develop hepatomegaly, steatosis and then hepatocellular adenomas (HCA), which can undergo malignant transformation. Recently, gene therapy has attracted attention as a potential treatment for GSD1a. In order to maintain long-term transgene expression, we developed an HIV-based vector, which allowed us to specifically express the human G6PC cDNA in the liver. We analysed the efficiency of this lentiviral vector in the prevention of the development of the hepatic disease in an original GSD1a mouse model, which exhibits G6Pase deficiency exclusively in the liver (L-G6pc−/− mice). Recombinant lentivirus were injected in B6.G6pc<sup>lox/lox</sup> x<sup>lox</sup>. SA<sup>creERT2/w</sup> neonates and G6pc deletion was induced by tamoxifen treatment at weaning. Magnetic resonance imaging was then performed to follow up the development of hepatic tumours. Lentiviral gene therapy restored glucose-6 phosphatase activity sufficient to correct fasting hypoglycaemia during 9 months. Moreover, lentivirus-treated L-G6pc<sup>−/−</sup> mice presented normal hepatic triglyceride levels, whereas untreated mice developed steatosis. Glycogen stores were also decreased although liver weight remained high. Interestingly, lentivirus-treated L-G6pc<sup>−/−</sup> mice were protected against the development of hepatic tumours after 9 months of gene therapy while most of untreated L-G6pc<sup>−/−</sup> mice developed millimetric HCA. Thus the treatment of newborns by recombinant lentivirus appears as an attractive approach to protect the liver from the development of steatosis and hepatic tumours associated to GSD1a pathology.
Introduction

Glycogen storage disease (GSD) type 1 is an inherited disease caused by the deficiency in glucose-6-phosphatase (G6Pase) activity (1–3). G6Pase catalyses the hydrolysis of glucose-6-phosphate (G6P) into glucose and inorganic phosphate, the terminal reaction in hepatic glucose production, common to glycogenolysis and gluconeogenesis. GSD type 1a (GSD1a, OMIM #232200) is caused by mutations affecting the catalytic subunit (G6PC) (4,5) of G6Pase. G6PC is specifically expressed in the liver, kidneys and intestine, which are the only organs capable of producing glucose in the blood (6–8). Thus patients affected by GSD1a are unable to produce glucose, leading to severe hypoglycaemia during fasting in association with lactic acidemia, hypertriglyceridaemia, hyperuricaemia and hypercholesterolaemia (2,3,9). Furthermore, G6Pase deficiency leads to the accumulation of G6P, glycogen and triglycerides (TG) in the liver, resulting in hepatomegaly and steatosis. The long-term complications in hepatic G6Pase deficiency include focal nodular hyperplasia and, more often, hepatocellular adenoma (HCA), with a risk of transformation into hepatocellular carcinomas (HCC) (9–11). HCAs predominantly develop during and after puberty, and more than 70% of adult GSD1 patients have multiple HCAs. In addition, patients with GSD1a also present nephropathy and intestinal disorders, which appear with time (3,9).

The current treatment of GSD1a focuses on the prevention of hypoglycaemia and lactic acidemia through frequent meals and ingestion of uncooked cornstarch during the night (12). However, the underlying pathology remains uncorrected and the dietary therapy fails to prevent serious long-term complications, such as hepatic tumours. Thus tumour resection or liver transplantation are recommended if the tumours are associated with serious compression or haemorrhage or show signs of malignant transformation into HCC (12,13). Recently, gene therapy has attracted attention as a potential treatment for GSD1a, because the liver is well suited for gene transfer. Indeed, the liver has been the target for potential treatment for GSD1a, because the liver is well suited for gene transfer. In contrast, blood glucose reached normal levels in HIV-G6PC mice (encoded GFP) was injected as control. As previously described (32), L-G6pc−/−/mice showed hypoglycaemia after 6 h of fasting (Fig. 1A) (32). Indeed, blood glucose remained low in untreated L-G6pc−/− mice after 6 h of fasting, reaching about 50–60 mg/dl compared with 140–150 mg/dl in WT mice (Fig. 1A). In contrast, blood glucose reached normal levels in HIV-G6PC treated L-G6pc−/− mice from 2 months after G6pc deletion and throughout the course of the study, i.e. until 9 months (Fig. 1A). Concomitantly, biochemical analyses revealed that plasma TG were restored in lentivirus-treated mice (Fig. 1B). However, hypercholesterolaemia was not corrected by gene therapy (Fig. 1C).

Prevention of hepatic GSD1a pathology using lentiviral gene therapy

Usually the hepatomegaly is the first symptom detected in GSD1a. It is caused by a massive G6P accumulation, leading to excessive glycogen stores. Moreover, the hepatic G6Pase deficiency leads to TG accumulation in the hepatocytes, resulting in hepatic steatosis. As previously described (32), L-G6pc−/− mice showed enlarged livers associated with accumulation of G6P, glycogen and TG (Fig. 2). Interestingly, hepatomegaly was partially prevented in lentivirus-treated L-G6pc−/− mice (the liver accounted for 5.6 ± 0.1% of total body mass (BM)) compared with untreated L-G6pc−/− mice (the liver accounted for 9.4 ± 0.6% of total BM), without being normalized compared with WT mice (the liver accounted for 4.2 ± 0.1% of total BM) (Fig. 2A). This was associated with a substantial decrease in hepatic G6P overload (1.6 ± 0.1 µmol G6P/g of liver in lentivirus-treated compared with 4.0 ± 0.3 µmol G6P/g of liver in untreated L-G6pc−/− mice) (Fig. 2B). Moreover, lentivirus-treated L-G6pc−/− mice presented a decrease in hepatic glycogen content after 9 months of gene therapy (46.3 ± 2.6 mg glycogen/g of liver) compared with untreated L-G6pc−/− mice (69.8 ± 4.8 mg glycogen/g of liver) (Fig. 2C). Interestingly, hepatic steatosis was substantially prevented by lentiviral gene therapy.
therapy because hepatic TG content of lentivirus-treated L-G6pc−/− mice was lower than that of untreated L-G6pc−/− mice and not significantly different as that of the WT mice (Fig. 2D). Histological observations of the livers of untreated L-G6pc−/− mice confirmed a marked steatosis, with an enlargement of hepatocytes containing large lipid vacuoles, mainly in the periportal areas (Fig. 3Ab). Lentivirus-treated L-G6pc−/− mice exhibited large areas showing normal appearance of hepatocytes. However, some areas with enlarged hepatocytes were still observed (Fig. 3Ac). Moreover, serial section analyses performed in the liver of lentivirus-treated L-G6pc−/− mice revealed that hepatocytes expressing G6PC, detected by immunohistochemistry (Fig. 3Bb), presented a normal morphology with an absence of lipid vacuoles (Fig. 3Ba). In contrast, the areas with steatosis matched perfectly with G6pc−/− hepatocytes, which did not express G6PC (Fig. 3B). To confirm that hepatocytes expressing G6PC were corrected cells, we analysed the expression of human G6pc mRNA in the lentivirus-treated liver compared with that of the mouse G6pc mRNA of WT liver. As expected, the human G6pc mRNA was undetectable in untreated mice and was substantially expressed in lentivirus-treated mice. Quantitatively, its expression was 10-fold lower than that of endogenous mouse G6pc mRNA in the WT liver (Fig. 3C). It may be noted that the transcription of a truncated mouse G6pc mRNA (containing exons 1 and 2) was upregulated in L-G6pc−/− liver, as previously reported (32). Consistent with a decrease in glycogen stores and normalization of hepatic TG levels, none of the HIV-G6PC treated L-G6pc−/− mice (total of 11) developed hepatic tumours, as observed by magnetic resonance imaging (MRI) analysis after 9 months of gene therapy. In contrast, most untreated L-G6pc−/− mice (17 out of 24) had multiple millimetric hepatic lesions (1–4 mm in diameter) at the end of the experiment (Fig. 4A and B). Almost all lesions corresponded to HCA (Fig. 4C and E), with the exception of one
found in an untreated L-G6pc<sup>−/−</sup> mouse, which developed an HCC (Fig. 4D and F).

Mosaic restoration of G6PC in L-G6pc<sup>−/−</sup> livers after gene therapy

As previously observed, G6Pase was mainly expressed in the periportal area compared with the perivenous area of the liver in the WT mice (Fig. 5Aa and b). In agreement with the deletion of G6pc, G6PC protein was undetectable by immunohistochemistry in untreated L-G6pc<sup>−/−</sup> mice (Fig. 5Ac and d). Interestingly, G6PC was specifically localized in hepatocytes found in the close vicinity of blood vessels after 6 weeks post-injection in lentivirus-treated L-G6pc<sup>−/−</sup> mice; these hepatocytes exhibited high immunostaining for G6PC whereas a substantial proportion of the surrounding hepatocytes were G6PC-negative (Fig. 5Ae and f). After 9 months of gene therapy, lentivirus-treated L-G6pc<sup>−/−</sup> hepatocytes presented a patchy localization of the G6PC protein, in both the periportal and perivenous areas (Fig. 5Ag and h). Thus immunostaining of G6PC was observed in large and diffuse areas around vessels. The presence of large and diffuse G6PC positive areas 9 months after the lentiviral gene therapy was probably due to hepatocyte divisions. Indeed, lentivirus-treated L-G6pc<sup>−/−</sup> mice exhibited an increased number in transduced hepatocytes, from 8 ± 3.5% at 6 weeks to 18 ± 0.9 at 9 months after hepatocyte transduction (Fig. 5B). Moreover, the G6Pase activity in the liver represented about 25% of WT hepatic G6Pase activity at 9 months post-injection, respectively (Fig. 5C). Finally, it is noteworthy that the DNA transgene was detected in several tissues after lentiviral gene therapy because the viral vectors were injected into the bloodstream (Fig. 5D). Nine months after gene therapy, the viral vector copy numbers were determined in the livers of lentivirus-treated mice by qPCR and found to represent about 3 copies per 100 cells. As expected, the human G6PC transgene was mainly expressed in the liver, although we observed a small leak in the spleen (data not shown).

Discussion

Since the 1980s, dietary management to achieve suitable metabolic control has considerably improved the life expectancy of GSD1 patients, but various complications occur with aging. One of the main causes of morbidity and mortality in aging GSD1 patients derives from the development of HCA. Most patients develop many HCAs, which can grow, become hemorrhagic or malignant over time (10–12). In this context, liver gene therapy provides an attractive alternative to liver transplantation, which is currently the only effective therapy performed on the basis of serious impairment of metabolic control or risk of malignant transformation. To date, the most promising gene therapy approaches to treat GSD1a were focused on the use of AAV in order to achieve efficient in vivo gene transfer in the liver (17,18). However, AAV vectors rarely integrate into the host genome, remaining primarily in an episomal form, and thus providing prolonged transgene expression only in the absence of cell division (33). Even after delivery in adult mice, a gradual loss of the transgene expression and of the therapeutic effect may be expected because of the turnover (even low) of hepatocytes (34,35). In addition, because of some cross-reactivity of neutralizing antibodies across AAV serotypes, the re-injection of a second AAV different from the first one may be inefficient. In contrast to AAVs, lentiviral vectors efficiently integrate into the cellular
genome, providing long-term expression, in both quiescent cells and cells that are actively dividing (24). However, it appeared that lentiviral vectors transduce less efficiently the cells that are in the G0 state of the cell cycle, such as differentiated adult hepatocytes (14,24,36). Moreover, an immune response to the therapeutic protein could also preclude the long-term correction of the disease (24). New strategies have been devised to prevent the expression of the transgene in antigen-presenting cells and thus to bypass the immune response (24). It must be mentioned that a previous study using a feline lentivirus expressing the human G6PC cDNA was performed at a neonatal stage, to avoid an immune response against the transgene components (37). This allowed the authors to normalize blood glucose and to attenuate the hepatic disease (i.e. a decrease in glycogen accumulation) in the total knockdown G6pc mouse after a double neonatal virus administration. Here we report the use of a similar recombinant vector, i.e. an HIV-based lentiviral vector carrying the human G6PC cDNA under the liver-specific mTTR promoter. It is noteworthy that this study was performed in L.G6pc−/− mice after a double neonatal virus administration. We report the use of a similar recombinant vector, i.e. an HIV-based lentiviral vector carrying the human G6PC cDNA under the liver-specific mTTR promoter. It is noteworthy that this study was performed in L.G6pc−/− mice exhibiting a long life expectancy that allowed us to study the effect of the lentiviral treatment on tumour development, a question not addressed in the former study. Interestingly, the HCA development seems to be accelerated in mice treated with tamoxifen at the age of 3 weeks, compared with mice treated at adult age (6–8 weeks). Indeed, small hepatic nodules of about 3–4 mm in diameter were detected in most untreated L.G6pc−/− mice after 9 months of G6pc deletion, whereas only 20–30% L.G6pc−/− mice showed millimetric nodules when G6pc deletion was performed in adults (32). In our study, lentiviral gene therapy restored about 25% of basal hepatic G6Pase activity that enabled the treated L.G6pc−/− mice to maintain normal fasting blood glucose levels and to normalize hypertriglyceridaemia for 9 months. Moreover, lentivirus-treated L.G6pc−/− mice presented a decrease in hepatic glycogen stores and a normalization of TG contents, at 9 months after gene therapy. In addition, lentivirus-treated mice did not show hepatic lesions after 9 months while most of untreated L-G6pc−/− mice developed HCA at this time. It is noteworthy that a recent study reported the prevention of HCA in the total G6pc KO mice treated by an AAV approach, despite hepatomegaly and elevated hepatic glycogen stores (19). However, the AAV-treated G6pc KO mice exhibited no steatosis and had normal levels of hepatic TG (19). Taken together with the study here, these data suggest that the excessive lipid accumulation rather than the glycogen accumulation could be the cause of the long-term consequence of HCA developed in L-G6pc−/− mice. Interestingly, many evidence suggest that nonalcoholic fatty liver disease, which is characterized by hepatic steatosis, is associated with the development of hepatic tumours, without overt hepatic fibrosis or cirrhosis (38,39). Some hypothetical mechanisms include oxidative stress, adipocytokine functional disorder or chronic inflammation linked to lipo-toxicity (40). Further studies should be conducted to highlight the central role of fatty liver in tumour development. In our study, the prevention of HCA formation with lentiviral gene therapy is associated with the presence of large and diffuse areas expressing G6PC, representing about 20% of the liver surface (see Fig. 5Ag–h and B). This is probably due to the transgene...
integration into the host genome and transmission during hepatocyte divisions in the neonatal liver. The clonal expansion of transduced cells could be explained by a selective advantage to treated cells. However, long-term studies will be required to know whether the liver GSD1a disease is only improved for a while or whether it is cured definitely. Indeed, the rescue of a partial G6Pase activity in a low proportion of hepatocytes could only permit to maintain blood glucose level and will not cure the rest of hepatocytes, which will still be prone to hepatic tumour development with time. In contrast to monogenic diseases that involve a primary defect in a hepatic protein without significant parenchymal damage and hepatic injury, such as Crigler-Najjar disease or coagulation defects, the gene therapy approach of GSD1a has to correct a maximum of G6pc−/− hepatocytes in order to prevent at best the development of the hepatic disease. Indeed, Crigler-Najjar disease or coagulation defects only require the expression of only low levels of the related hepatic protein in the blood stream to treat or prevent the systemic illness manifestations.

Finally, in comparison to gamma retroviral LTR-driven vectors, lentiviral vectors present with favourable features like a potentially safer insertion profile. Thus our study supports the absence of genotoxicity of HIV1-derived lentiviral vectors in the liver (23,25). However, the residual genotoxicity risk associated with the preferential intragenic integration events represents the most obvious concern. The substantial flexibility of the lentiviral vector genome offers opportunities for redesigning sequences of the backbone as well as the therapeutic cargo to further reduce this risk.

In conclusion, we demonstrate here that the lentiviral gene therapy performed during the neonate period permits a strong expression of G6Pase, corrects fasting blood glucose levels and...

Figure 4. Tumour development in L-G6pc−/− mice. (A and B) Liver resection after 9 months of hepatic G6pc deletion. Arrows point nodules of about 3–4 mm of diameter. (C–F) Histological analysis by H&E staining of an HCA (C and E) and an HCC (D and F) developed in untreated L-G6pc−/− mice. (E) and (F) correspond to a higher magnification of the tumours observed in (C) and (D), respectively.
prevents the hepatic disease, including the HCA formation, for at least 9 months in L-G6pc−/− mice. However, more efficient approaches are still needed to improve all parameters, including glycogen stores. A specific and stronger hepatic expression of the transgene could be obtained by adding regulatory sequences to the mTTR promoter (30,41,42). Moreover, because the mTTR promoter is leaky permitting a weak expression in non-liver cells, in particular in antigen-presenting cells, the addition of mir142-3pT sequence in the vector backbone would be useful to prevent an immune rejection of transduced cells (27,43). In addition, it is interesting to note that the lentiviral vector gene therapy permits the transduction of the three organs affected by GSD1a, i.e. the liver, kidney and intestine (see Fig. 5D). This indicates that this approach might be suitable as a possible treatment of the kidney or intestine diseases if combined with the use of an appropriate promoter. Because a neonatal intervention in a clinical setting may drastically improve the phenotype in the most critical years of an infant development, the lentiviral gene therapy appears as an attractive approach for GSD1a treatment.

Materials and Methods
Preparation of recombinant vectors encoding human G6PC
We produced an HIV-based lentiviral vector carrying the human G6PC cDNA (or the GFP cDNA as control) under the control of the mTTR promoter, fused to a synthetic hepatocyte-specific enhancer that specifically directs transcription to the liver (28), that specifically directs transcription to the liver. High-titre lentiviral vector stocks were generated as previously described by calcium phosphate-mediated transient transfection of three
plasmids: the vector plasmid, the packaging plasmid psPAX2 and the vesicular stomatitis virus G protein (VSV G) envelope protein-coding plasmid pMD2G (44). These self-inactivating transfer vectors harboured the cis-acting cPPT/CTS from HIV-1, which facilitates the nuclear translocation of preintegrative vector complexes (45,46) and the post-transcriptional regulatory element from the woodchuck hepatitis virus, which increase transgene expression (47) and was inserted downstream of the transgene (Supplementary Material, Fig. S1). The titres of the viral preparations were determined as previously described (28) and were in the range of $1 \times 10^7$ transducing units/ml.

AAV and lentiviral vector administration into mice

Two-day old B6.G6pcex3lox/ex3lox.SAcreERT2/w mice were infused via the temporal vein with 50 μl of HIV-mTTR-hGFP virus ($n = 11$) or HIV-mTTR-hGFP virus containing $4 \times 10^7$ to $10 \times 10^7$ transducing units in phosphate-buffered saline. Mice of the untreated group ($n = 24$) corresponded to HIV-mTTR-hGFP injected mice.

Generation of liver-specific G6pc knock-out mice

The deficiency in G6Pase was obtained by a specific deletion of G6pc exon 3 in the liver, using a CRE-lox strategy (32). B6. G6pcex3lox/ex3lox SAcreERT2/w and C57Bl/6J mice (Charles Rivers Laboratories, L’Arbresle, France) of both genders were injected intraperitoneally at 15–21 day old with 0.5 mg of tamoxifen every 4 day for consecutive days, in order to obtain L-G6pc−/− and wild-type (WT) mice, respectively. Mice were housed in the animal facility of Lyon University under temperature controlled (22°C) conditions and with a 12/12 h light/dark cycle. Mice had free access to water and standard chow diet. Fasted mice were provided with continuous access to water. All the procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. The animal care committee of University Lyon 1 approved all the experiments.

Magnetic resonance imaging

MRI examinations were performed 5, 7 and 9 months after G6pc deletion on anaesthetized mice on an horizontal 4.7T Biospec system (Bruker, Ettlingen, Germany) (32). T2-weighted contrast images were acquired in the axial plane with a fat suppressed rapid acquisition with relaxation enhanced (RARE) sequence with the following parameters: repetition time >6 s; 38.2 ms echo time; $30 \times 30 \text{ mm}^2$ field of view; 256 × 192 matrix, 32 slices of 0.7 mm thick; RARE factor = 4; two averages; 98 Hz of pixel bandwidth. A trained radiologist who has over 20 years of experience analysed the MR images to detect nodules in vivo.

Biochemical assays

Blood glucose was followed after 6 h of fasting with an Accu-Chek Go glucometer (Roche Diagnostics, Meylan, France). Blood was withdrawn by submandibular bleeding using a lancet. Plasma TG and cholesterol were determined with Biomérieux colorimetric kits (Marcy l’Etoile, France). Mice were killed by cervical dislocation. The liver was immediately removed, weighed and snap-frozen using tongs previously chilled in liquid N2 and stored at –80°C. All tissues were analysed macroscopically. Hepatic glycogen determinations were carried out on frozen tissue homogenates according to the procedure previously described (48). Total hepatic lipids were extracted by the method of Bligh and Dyer (49), and triglyceride content was measured using a Biomérieux colorimetric kit. Hepatic G6Pase activity was assayed at maximal velocity (20 mmol/l of G6P) at 30°C, as previously described (50).

Gene expression

Total RNAs were extracted from livers with TRIzol reagent (Invitrogen life technology, St Aubin, France), according to the manufacturer’s instructions. Reverse transcription was performed using the kit QuantiTect Reverse transcription (Qiagen France, Courtabeuf). Quantitative polymerase chain reaction (PCR) was performed using the Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Marnes-la-Coquette) in the CFX Manager Realplex (Bio-Rad). Endogenous G6pc exon 1-exon 3 mRNA fragment was amplified using exon 1 mG6pc sense 5′-TTACCAAGAC TCCCCAGACTG3′ and exon 2 mG6pc antisense 5′-GACCTGTGTC TTGATATGTCG3′. The specific human G6PC exon 4-exon 5 mRNA fragment was amplified using exon 2 mG6pc sense 5′-AGCG TCCCATCTGTTGGT TT3′ and exon 3 mG6pc antisense 5′-GGTC GGCTTATCTTCCCCTG3′. The mouse ribosomal protein L19 transcript (Rpl19) was used as housekeeping gene (32). Calculations were based on the comparative cycle threshold method (2−ΔΔCt).

Histological analysis

A piece of the liver and hepatic nodules were fixed in 10% formalin for histological analyses and embedded in paraffin. Serial sections (4 µm thick) were cut and stained with haematoxylin and eosin (H&E) staining. Immunohistochemical analyses were performed as previously described, using a purified rabbit anti-G6PC antibody (dilution 1:1000) (51). The slides were examined under a Coolscope microscope (Nikon Instruments, New York, USA). The G6PC-positive cell index was calculated and expressed as the percentage of positive stained area against total analysed area.

The following criteria were used to identify HCA and HCC. HCA were defined as nodules, visible at macroscopic examination, well circumscribed, expansive, compressing the adjacent parenchyma. They were made of well-differentiated hepatocytes, usually larger than normal hepatocytes, with low nuclear-cytoplasmic ratio, eosinophilic, clear or vacuolated cytoplasm, and enlarged nuclei with well visible nucleoli. The normal trabecular architecture was retained; plates were regular and one to two cells thick. No connective septa were present. Mitotic figures were rare. In some cases, smaller basophilic, proliferative hepatocytes could be observed, usually at the periphery of the nodule; cell atypia might be present but only focally; no evidence of local tissue invasion or angioinvasion was detected. HCC was defined by the presence of architectural and cellular atypia. The architecture was mainly trabecular but plates were irregular, usually 2- to 4-cell thick branched and separated by dilated and capillarized sinusoidal vessels. Hepatocytes were of variable size, but were frequently characterized by low nuclear-cytoplasmatic ratio and basophilic cytoplasm. Nuclear atypia was frequent. Mitoses were numerous. Connective septa could be present. Tissue invasion and/or angioinvasion might be detected.

Supplementary Material

Supplementary Material is available at HMG online.

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