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ORIGINAL RESEARCH PAPER

The chimeric cytokine Hyper-IL-6 enhances the efficiency of lentiviral gene transfer in hepatocytes both in vitro and in vivo

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Abstract Lentiviral vectors have been used for gene transfer into the liver but their ability to efficiently transduce quiescent hepatocytes remains controversial. Lentivirus-mediated gene transfer is more efficient in cycling cells. We determine the effect of H-IL6 in the lentiviral transduction. The lentiviral vector was used to transduce HepG2 cells and mice liver cells, previously treated with H-IL6. The highest transduction level was observed in HepG2 cells treated with 30 ng/mL H-IL6 and in the mice that received 4 μg H-IL6. Our results suggest that H-IL6 is an inducer of lentiviral gene transfer into the liver cells without any toxicity.

Keywords Gene therapy · Hepatocytes · H-IL6 · Lentiviral vectors · Transduction efficiency

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Introduction

The liver is an interesting target for gene therapy for its central role in many diseases. Several innate and acquired diseases are caused by defects in liver expressed genes (Schmitz et al. 2002). Moreover, the liver has been evaluated as a potential depot organ due to the production of a variety of therapeutic proteins that act systemically (Beck 2007).

Besides the gene therapy, gene transfer methods are also important to basic research, in order to understand gene's function and molecular pathophysiology. Many gene transfer vector systems already developed could be used to target the liver. Viral vectors as retrovirus (Kaleko et al. 1991; Kitten et al. 1997), adenovirus (Schiedner et al. 1998; Yao et al. 1996) and adeno-associated virus (Nakai et al. 1998; Snyder et al. 1997) have been used showing high transduction efficiency in vitro. However, their efficiency in vivo is limited and it need to be improved to attain therapeutic levels.

Among the viral vectors, retroviruses, have been widely used due their safety and ability to integrate into host cell genome, allowing long-term sustained gene expression (Kay et al. 2001). However, retroviral vector for liver-direct gene therapy is limited because cell cycling is an essential requirement for DNA integration (Miller et al. 1990).

Lentiviruses represent a class of retrovirus with infection potential for both cycling and noncycling cells (Emerman 2000), and have shown to stably

transduce a wide variety of quiescent cells in vitro and in vivo (Kafri et al. 1997; Miyoshi et al. 1999; Naldini et al. 1996a, b). However, it is possible that lentiviral vectors require some level of cell cycling in vivo to enhance transduction in cells such hepatocytes (Park et al. 2000).

Hyper-IL6 (H-IL6) is a fusion protein consisting of human IL-6 and the human soluble receptor sIL-6R connected by a flexible polypeptide chain (Fischer et al. 1997), which was shown to have a high biological activity in concentrations 100–1,000 fold lower than IL-6 alone or in combination with sIL-6R. H-IL6 has been shown to be highly active to induce liver proliferation (Hecht et al. 2001).

We have used a third generation HIV-based lentiviral vector pSIN.cPPT-SFFV/GFP-WPRE. This vector encodes GFP as report gene, and it was used to transduce hepatocytes both in vivo and in vitro, either in the presence or the absence of H-IL6. This study shows that in the presence of H-IL6 the lentiviral transducted is markedly enhanced in hepatocytes both in vivo and in vitro.

Methods

Lentiviral vector

The vector used in this study was the pSIN.cPPT-SFFV/GFP-WPRE lentiviral vector (SWE), which codifies for enhanced green fluorescent protein (EGFP), under control of the SFFV promoter (Fig. 1) obtained from Adrian Thrasher, London, England (Demaison et al. 2002).

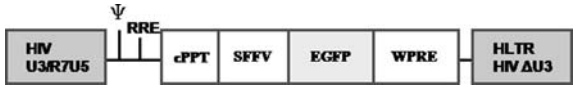

Fig. 1 Schematic diagram depicting the pSIN.cPPT-SFFV/GFP-WPRE (SEW) lentiviral vector design. In the SEW the internal promoter SFFV drives the EGFP expression. The WPRE and cPPT have been incorporated to the vector to improve vector performance. The Rev-responsive element (RRE) for the provirus RNAs transport to cytoplasm and the packing signal (Ψ) are shown. This lentiviral vector is self-inactivating, it has a deletion in the 3′LTR (ΔU3). Abbreviations: SFFV, slpeen focus forming virus; EGFP, enhanced green fluorescent protein; SIN, self-inactivating; WPRE, woodchuck post-transcriptional regulatory element; cPPT, central polypurine tract element

Lentiviral vector production

The DNA vectors were introduced into 293T cells by a triple cotransfection using the lentiviral vector SEW, the packaging construct pCMVR8.91 (Mastromarino et al. 1987; Zufferey et al. 1997), and the envelope plasmid vesicular stomatitis virus glycoprotein pMD2.VSVG (Follenzi and Naldini 2002a, b; McGlynn et al. 1996). The viruses were generated by calcium phosphate-mediated transient transfection of a three-plasmid system as described previously (Follenzi and Naldini 2002b; Kalsheker et al. 2002). Sixteen hours after transfection, the supernatants were replaced by fresh medium and cells were incubated for additional 48 h. The culture supernatants containing pseudovirions were concentrated by ultracentrifugation (50,000 μg, 2 h, 4°C), and pellets were resuspend in PBS (Invitrogen) containing 1% human serum albumin (Red Cross Blood Donor Service Baden Wuerttemberg).

Virus titers were determined by seeding 5×10^4 293T cells per well in a 24 well plate, and, after twenty four hours of culture the cells were infected with serially diluted concentrated virus and supplemented with 1 U polybrene ml^{-1} (Sigma).

After 72 h of incubation, transduced cells were detected by EGFP expression using fluorescence-activated cell sorting (FACS) on a FACS Calibur flow cytometer (BD Biosciences).

Cell culture

Human embryonic kidney epithelial cells 293T (DSMZ ACC 305) and human HepG2 hepatocyte carcinoma cells (DSMZ ACC 180) were cultured in DMEM with 10% (*v*/*v*) fetal bovine serum. All cultures were grown as monolayer in humidified incubators, at 37°C in an atmosphere of 5% (v/v) CO_2.

In vitro lentiviral transduction of HepG2 cells

One day before the assay, HepG2 cell line was seeded at 2×10^5 cells/well in 24-well plates. Cells were transduced with SEW lentiviral vector at a multiplicity of infection (MOI) of 1. After centrifugal inoculation (1,250 g, 90 min, 32°C) and subsequent

Table 1 In vivo experiment schema

Mice group	H-IL 6 (day 0)	PBS (day 0)	SEW lentiviral vector (day 1)
1	4 μg (i.p.)/100 μl	–	10^8 (i.v.)
2	4 μg (i.p.)/100 μl	–	10^9 (i.v.)
3	2 μg (i.p)/100 μl	–	10^8 (i.v.)
4	2 μg (i.p)/100 μl	–	10^9 (i.v.)
5	–	100 μl (i.p)	10^8 (i.v.)
6	–	100 μl (i.p)	10^9 (i.v.)
7	4 μg (ip)/100 μl	100 μl (i.p)	–

SEW, lentiviral vector SEW; IP., intraperitoneal injection; IV., intravenous injection

culture for 16 h, the culture medium was changed by fresh medium in the presence (5, 10, 20, 30, 40 and 50 ng ml^{-1}) or absence of H-IL6. The cells were incubated for additional 48 h at 37°C. Transduction efficiency was determined by EGFP expression using flow cytometric analysis. All experiments were performed in triplicate.

Lentivirus injection into NOD-SCID mice

Twenty-one female NOD-SICD mice were used for the in vivo experiment. The mice were divided into seven groups according to the treatment administered (Table 1). After seven days, the mice were sacrificed. Blood and livers were collected for analyses to determine the level of ALT (alanine aminotransferase) as well as AST (aspartate aminotransferase) and the number of integrated lentiviral copies.

Toxicity studies

Evaluation of liver toxicity was done by measurements of serum ALT and AST activities using a previous described method (Henry et al. 1960) on RCC Laboratory (Füllinsdorf, Germany).

Lentiviral copy number quantification

The DNA was extracted from hepatic cells using DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. The number of lentiviral provirus copies per genome were quantified by Real Time qPCR using the GAG primers forward (5′-GGA GCT AGA ACG ATT CGC AGT TA-3′) and reverse (5′-GGT TGT AGC TGT CCC AGT ATT TGT C-3′), and the GAG probe (5′-(FAM)- ACA GCC TTC TGA TGT TTC TAA CAG GCC AGG -(Eclipse Dark Quencher)-3). For normalization of the provirus copy number we quantified the number of mouse β-actin gene using the mβ-actin primers and probe: forward (5′-AGA GGG AAA TCG TGC GTG AC-3′), reverse (5′-CAA TAG TGA CCT GGC CGT-3′), and mβ-actin probe (5′-(Yakima Yellow)-CAC TGC CGC ATC CTC TTC CTC CC-(Eclipse Dark Quencher)-3′). The primers and double dye-oligonucleotide conjugated probes were purchased from Eurogentec (Seraing). Reactions were carried out according to manufacturer's instructions. It was used two standards, the first one with the diluted gene transfer vector (from 1×10^6 copies to 1 copy/reaction in GeneAmp 1 × PCR buffer containing 100 ng mouse genomic DNA μl^{-1}). The second one with the diluted HepG2 DNA (from 2×10^6 to 10 cells/reaction in the same buffer). The results were analyzed using ABI Prism 7700 sequence detection system (PE-Applied Biosystems). All samples were done in duplicate. The determination of proviral integration was done by dividing the total proviral genomic copies by half of the number of β-actin copies.

Results

In this study, we used HyperIL-6 to activate hepatocyte proliferation in vitro and in vivo with the aim to improve lentiviral integration.

HepG2 cells were transduced with lentiviral vector SEW at MOI 1 with different H-IL6 doses and analyzed for EGFP expression (Fig. 2).

20 and 30 ng H-IL6 ml^{-1} were the most effective to induce SEW transduction into HepG2 cells, which showed 14% and 23% of EGFP positive cells respectively. 10 and 5 ng H-IL6 ml^{-1} did not alter the efficiency of lentiviral transduction. Also, higher concentrations of H-IL6 (40 and 50 ng ml^{-1}) did not improve lentiviral transduction. At these doses we could observe that the number of cells was lower compared with minor doses suggesting a toxicity effect.

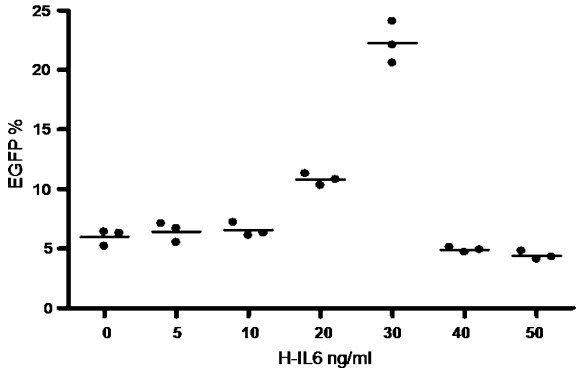

Fig. 2 H-IL-6 cytokine improves the lentiviral vector transduction efficiency in vitro into hepatoma cell line. Cells were transduced for 16 h with SEW (MOI, 1). Then, the cells were treated with or without H-IL-6. At 48 h after treatment, the transduction efficiency was measured by EGFP %. Values are shown as means SD of triplicate cultures

The second aim of this study was to use the H-IL6 to improve the transduction efficiency in vivo. First, we investigated whether H-IL6 or delivered dose of lentiviral vector could induce some toxicity to the mice. Our date showed that the intraperitoneal injection of 2 or 4 μg of H-IL6 in NOD-SCID mice as well as the lentiviral dose at 10^8 and 10^9 were not toxic as indicated by the level of AST and ALT. The AST and ALT plasma levels were similar at all mice groups (Fig. 3).

Second, we analyzed the number of provirus copies in relation to H-IL6 and lentiviral doses. The highest SEW copy number was obtained in animals treated with 4 μg of H-IL6. These mice groups (1 and 2) showed about 13 times more SEW copies in comparison to those groups that received 2 μg H-IL6 (group 3 and 4) or to those that did not receive H-IL6 (group 5 and 6). The number of SEW copies found in the mice groups 1 and 2 (with 4 μg H-IL6) were in average 6,200 and 8,500 lentiviral copies/100000 liver cells, respectively. The treatment with 2 μg of H-IL6 resulted in few lentiviral integrated copies which were similar to those found in the absence of H-IL6. As shown in Fig. 4 the number of SEW copies in the mice groups 3 and 4 were on average 233 lentiviral copies/100000 liver cells and in the mice groups 5 and 6 were in average 910 lentiviral copies/100000 liver cells. In all mice groups, the increase of 10 times on lentiviral titer did not improve the number of copies in the liver cells (Fig. 4). In conclusion, our data indicate that 4 μg H-IL6 improve the rate of lentivirus integration in the liver cells without any toxicity.

Discussion

Several studies have shown that lentiviral vectors are capable of transducing non-dividing cells into different organs (Kafri et al. 1997; Naldini et al. 1996a, b). However, no previous studies have directly established the cell-cycle status of any transduced cell type at the time of vector administration in vivo. In vitro studies using wild-type HIV or HIV-based vectors have shown that, in some cases, cell-cycle activation is required for infection. (Korin and Zack 1998; Sutton et al. 1999).

This was the first study to show the improvement of lentiviral transduction efficiency in vitro and in vivo after treatment with the chimeric cytokine hyper-IL-6.

Our in vitro date with different H-IL6 concentrations showed a high transduction rate into HepG2 cells treated with 30 ng H-IL6 ml^{-1}. However, higher H-IL6 concentrations (40 and 50 ng ml^{-1}) were toxic, which led cells to die. Selden et al. (2007) have also used a third-generation lentiviral vector at MOI 1 and reported that about 15% of human hepatocytes could

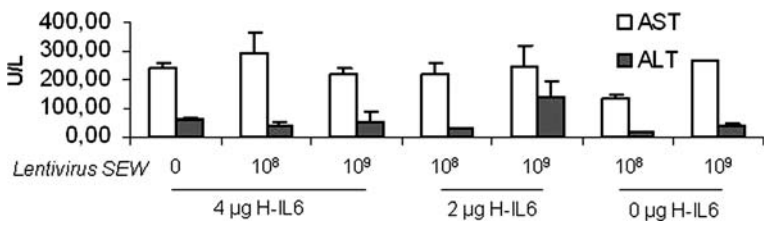

Fig. 3 H-IL-6 cytokine did not cause any liver injury to the mice. Comparison of serum AST (white bars) and ALT (black bars) levels in mice (n = 3 per group), 7 days after receiving treatment with H-IL-6 (2 and 4 μg) and infusion with SEW vector (MOI, 10^8 and 10^9). No evidence of liver damage was found in either untreated or H-IL-6 treated animals. The levels of AST and ALT are similar to both groups

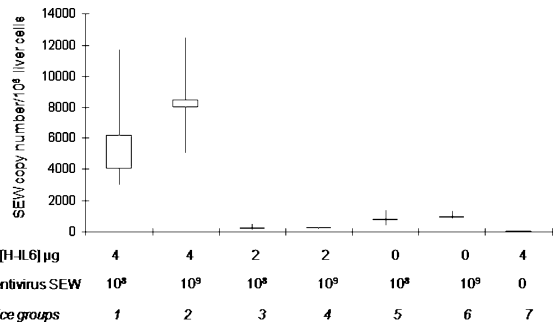

Fig. 4 H-IL-6 cytokine improves in vivo the SEW lentiviral copy number into the liver cells. Mice were treated with different doses of H-IL6 and received SEW lentiviral particles (10^8 or 10^9). After seven days the mice were killed and the real-time PCR analysis was performed on DNA isolated from the liver. The number of lentiviral copies was obtained from known copies of positive control plasmid diluted into negative control mouse liver DNA. Results are expressed as number of copies/cell. Means ± SD, $n = 3$, are shown

be transduced in monolayer culture at the presence of hepatocyte growth factors (Selden et al. 2007). Our work indicates that at the same MOI, the treatment with 30 ng ml^{-1} of H-IL6 resulted in more than 20% of transduced hepatocytes in vitro.

At the in vivo experiment, liver samples of NOD-SCID mice infected with SEW lentiviral vector (10^8 or 10^9 viral particles) and injected with 4 µg H-IL6 improved the lentiviral integration efficiency in comparison to the mice that received 2 µg. These data showed a higher transduction efficiency using H-IL6, when compared to other previous results that used a surgical method (Ohashi et al. 2002; Park et al. 2000) or direct administration of lentivirus without any pre-treatment (Follenzi et al. 2004).

Also, this study shows that it is possible to improve the lentiviral transduction with the use of H-IL6 without any toxicity for the mice. This has implications for liver gene therapy designing, which allows the reduction of lentiviral MOIs.

For therapeutic use, the ability to reduce the viral load for a given level of expression confers obvious advantages as increased safety and practicality of manufacturing protocols. Moreover, low virus doses reduce gene therapy costs, cellular toxicity and the immune system in vivo activation. All these advantages could potentially be realized by the addition of H-IL6 to lentiviral transduction protocols.

The lentiviral mediated gene delivery has a lifelong expression potential due to its efficient integration into target cells and significant insert DNA capacity. Nevertheless, the long-term safety of in vivo lentiviral vector administration needs further investigation; the findings reported here highlight the potential use of H-IL6 for liver-based gene therapy.

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References

Beck M (2007) New therapeutic options for lysosomal storage disorders: enzyme replacement, small molecules and gene therapy. Hum Genet 121:1–22

Demaison C, Parsley K, Brouns G, Scherr M, Battmer K, Kinnon C, Grez M, Thrasher AJ (2002) High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum Gene Ther 13:803–813

Emerman M (2000) Learning from lentiviruses. Nat Genet 24:8–9

Fischer M, Goldschmitt J, Peschel C, Brakenhoff JP, Kallen KJ, Wollmer A, Grotzinger J, Rose-John S (1997) I. A bioactive designer cytokine for human hematopoietic progenitor cell expansion. Nat Biotechnol 15:142–145

Follenzi A, Battaglia M, Lombardo A, Annoni A, Roncarolo MG, Naldini L (2004) Targeting lentiviral vector expression to hepatocytes limits transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. Blood 103:3700–3709

Follenzi A, Naldini L (2002a) Generation of HIV-1 derived lentiviral vectors. Methods Enzymol 346:454–465

Follenzi A, Naldini L (2002b) HIV-based vectors. Preparation and use. Methods Mol Med 69:259–274

Hecht N, Pappo O, Shouval D, Rose-John S, Galun E, Axelrod JH (2001) Hyper-IL-6 gene therapy reverses fulminant hepatic failure. Mol Ther 3:683–687

Henry RJ, Chiamori N, Golub OJ, Berkman S (1960) Revised spectrophotometric methods for the determination of glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and lactic acid dehydrogenase. Am J Clin Pathol 34:381–398

Kafri T, Blomer U, Peterson DA, Gage FH, Verma IM (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat Genet 17:314–317

Kaleko M, Garcia JV, Miller AD (1991) Persistent gene expression after retroviral gene transfer into liver cells in vivo. Hum Gene Ther 2:27–32

Kalsheker N, Morley S, Morgan K (2002) Gene regulation of the serine proteinase inhibitors alpha1-antitrypsin and alpha1-antichymotrypsin. Biochem Soc Trans 30:93–98

Kay MA, Glorioso JC, Naldini L (2001) Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat Med 7:33–40

Kitten O, Cosset FL, Ferry N (1997) Highly efficient retrovirus-mediated gene transfer into rat hepatocytes in vivo. Hum Gene Ther 8:1491–1494

Korin YD, Zack JA (1998) Progression to the G1b phase of the cell cycle is required for completion of human immunodeficiency virus type 1 reverse transcription in T cells. J Virol 74:3161–3168

Mastromarino P, Conti C, Goldoni P, Hauttecoeur B, Orsi N (1987) Characterization of membrane components of the erythrocyte involved in vesicular stomatitis virus attachment and fusion at acidic pH. J Gen Virol 68:2359–2369

McGlynn LK, Mueller CR, Begbie M, Notley CR, Lillicrap D (1996) Role of the liver-enriched transcription factor hepatocyte nuclear factor 1 in transcriptional regulation of the factor V111 gene. Mol Cell Biol 16:1936–1945

Miller DG, Adam MA, Miller AD (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 10:4239–4242

Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE (1999) Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. Science 283:682–686

Nakai H, Herzog RW, Hagstrom JN, Walter J, Kung SH, Yang EY, Tai SJ, Iwaki Y, Kurtzman GJ, Fisher KJ, others (1998) Adeno-associated viral vector-mediated gene transfer of human blood coagulation factor IX into mouse liver. Blood 91:4600–4607

Naldini L, Blomer U, Gage FH, Trono D, Verma IM (1996a) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci USA 93:11382–11388

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996b) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263–267

Ohashi K, Park F, Kay MA (2002) Role of hepatocyte direct hyperplasia in lentivirus-mediated liver transduction in vivo. Hum Gene Ther 13:653–663

Park F, Ohashi K, Chiu W, Naldini L, Kay MA (2000) Efficient lentiviral transduction of liver requires cell cycling in vivo. Nat Genet 24:49–52

Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S (1998) Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. Nat Genet 18:180–183

Schmitz V, Qian C, Ruiz J, Sangro B, Melero I, Mazzolini G, Narvaiza I, Prieto J (2002) Gene therapy for liver diseases: recent strategies for treatment of viral hepatitis and liver malignancies. Gut 50:130–135

Selden C, Mellor N, Rees M, Laurson J, Kirwan M, Escors D, Collins M, Hodgson H (2007) Growth factors improve gene expression after lentiviral transduction in human adult and fetal hepatocytes. J Gene Med 9:67–76

Snyder RO, Miao CH, Patijn GA, Spratt SK, Danos O, Nagy D, Gown AM, Winther B, Meuse L, Cohen LK, others (1997) Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet 16:270–276

Sutton RE, Reitsma MJ, Uchida N, Brown PO (1999) Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. J Virol 73:3649–3660

Yao SN, Farjo A, Roessler BJ, Davidson BL, Kurachi K (1996) Adenovirus-mediated transfer of human factor IX gene in immunodeficient and normal mice: evidence for prolonged stability and activity of the transgene in liver. Viral Immunol 9:141–153

Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15:871–875