Efficient delivery of human clotting factor IX after injection of lentiviral vectors *in utero*

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ABSTRACT

AIM: To explore gene transfer feasibility for human clotting factor IX (hFIX) mediated by recombinant lentivirus *in utero*. **METHODS:** ICR mice fetus at 17-19 d gestation were received lentiviral vectors carrying hFIX cDNA under the control of liver specific promoter by intrahepatic injection. The expression and distribution of hFIX cDNA and possible immune responses against the hFIX were assessed by ELISA, PCR, RT-PCR, and immunohistochemistry, respectively. **RESULTS:** The serum hFIX protein were detected at different time points in all newborn mice, the highest level of hFIX was 50 μg/L and lasted for more than 30 d. Anti-hFIX antibody was not detected. hFIX cDNA was detected in liver, spleen, and heart. The expression of hFIX cDNA was only detected in liver. Besides, no germ line transmission was found at DNA and RNA levels, and no side effect associated with gene transfer was detected. **CONCLUSION:** The efficient delivery of hFIX can be achieved by prenatal gene transfer. It thus shows the feasibility of gene therapy for hemophilia *in utero*.

INTRODUCTION

Hemophilia B is the severe bleeding disorder caused by the absence of functional coagulation factor IX, and can potentially be treated by sustained secretion of functional IX after *in vivo* viral gene transfer[1,2]. Gene transfer to the developing fetus offers several potential advantages over postnatal gene transfer[3-5]. Firstly, *in utero* gene therapy may allow for the correction or prevention of genetic diseases prior to the onset of phenotypic abnormalities. Secondly, the rapid expanding pool of fetal stem cells may be more efficiently transduced than that of the quiescent adult stem cell population. Thirdly, organs inaccessible later in life may be transduced during earlier stages of development. Finally, fetal immune immaturity may permit induction of immune tolerance to vector and transgene.

Developing vector systems for utero gene transfer presents special problems. Lentiviral-mediated gene delivery system is currently being paid a great deal of attention as an innovative tool for their ability to deliver therapeutic transgenes into a wide variety of difficult-to-transfect/transduce target tissues (brain, hematopoietic system, liver, lung, and retina) without eliciting significant humoral immune responses[6]. Lentiviral vectors have been used for gene transfer into the liver, but the ability of these vectors to efficiently transduce quiescent hepatocytes remains low efficiency. It has been reported that lentiviral-mediated gene transfer is greatly enhanced when delivered during hepatocellular proliferation[7]. Fetuses are in early period of development. The unique physiological characteristics make them more suitable for lentiviral-mediated gene transfer and

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expression *in vivo*, since numerous tissue cells are undergoing rapid growth and proliferation[8]. Mackenzie *et al*[9] reported that lentiviral vectors provided high-efficiency, long-term gene expression in the murine fetus with the distribution of transduction dependent upon the mode of administration and the vector pseudotype. However, the delivery of hFIX after injection of lentiviral vectors *in utero* has not yet been reported.

For the sake of exploring and developing a lentiviral-mediated, effective fetal gene therapy approach for hemophilia B, we constructed recombinant lentiviral vector FAXW in which the hFIX cDNA was driven by liver specific promoter. The expression and distribution of hFIX, as well as host immune response were investigated. Our results provided a useful information for lentiviral-mediated gene therapy for hemophilia B *in utero*.

MATERIALS AND METHODS

Reagents Various restriction enzymes and other reagents (T4 DNA ligase, dNTP, Taq DNA polymerase, MMLV reverse transcriptase, etc) were purchased from New England Biolabs. Lentiviral vector C-FUW, packaging plasmids CMVΔR8.2 and envelope plasmids VSV-G were kindly provided by Dr Lois of California Institute of Technology, USA. 293T cell line was provided by Dr Wei-guo JIA of University of British Columbia, Canada. Nonpregnant pathogen-free ICR male and female mice were purchased from BK Company of Shanghai, which were housed and inbred within a sterile environment at the Animal Center of Second Military Medical University (Shanghai, China). Mouse anti-human FIX monoclonal antibody 3A6 was provided by Dr Yoshioka from Nara Medical College of Japan. Rabbit anti-human FIX antiserum and peroxidase-conjugated goat anti-rabbit IgG antiserum were purchased from CalobioChem Company. PCR primers for hFIX were 5'-TATGCAGC-GCGCGTGAACA-3' and 5'-GGGACACCAACATTCA-TAGG-3'; β-actin primers were: 5'-CCTTCCTGTGCA-TGGAGTCCT-3' and 5'-GGAGCAATGATCTTGACT-TC-3'; primers were synthesized by Jikang Biology Engineering Company, Shanghai.

Construction of lentiviral vectors and virus production hFIX cDNA was inserted into *Eco*RI site of lentiviral vector C-FUW to generate recombinant lentiviral vector FUXW containing hFIX cDNA driven by ubiquitin-C promoter. The ubiquitin promoter of FUXW was replaced with ABP liver specific promoter to generate recombinant lentiviral vector FAXW (Fig 1). The identity of construct were confirmed by restriction enzyme mapping and DNA sequence analysis as our previously described[10]. Lentiviral vectors were produced in 293T cells by calcium phosphate mediated transient transfection of three plasmids: one transfer vector, the packaging vector and the VSV-G expression plasmid, and purified by ultracentrifugation as previously described[11]. Titers of virus were estimated by RNA dot blot analysis.

Injection of mouse fetus Animals were set up in breeding pairs and vaginal plug dates were recorded as d 0 of gestation. On d 16-18 of gestation, pregnant females were anaesthetized by an intraperitoneal injection of 2.5 % tribromoethanol in tertamyl alcohol (Sigma-Aldrich) diluted in phosphate-buffered saline (PBS). Transuterine, intrahepatic injection was performed as previously described[9]. A dose of 8×10^7 virus genome (vg) of recombinant lentivirus was delivered in 10 μL. Following injection, the uterus was returned to the abdominal cavity and the abdominal wall was closed. Control animals were injected PBS. We sacrificed the animals at various time points between 5 d and 1 month for processing.

Detection of factor IX and antibodies Expression of hFIX was detected by ELISA. The first, second and third antibodies were mouse anti-hFIX monoclonal antibody 3A6, rabbit anti-hFIX antibody, and HRP conjugated goat anti-rabbit IgG. ABTS was the substrate used to colorize. Absorbance was read at 410 nm with a microplate reader. The expression level was calculated according to the standard curve derived from

Fig 1. Recombinant lentiviral vector FAXW. HIV-1 flap: human immunodeficiency virus-1 flap element; U3: deletion in the U3 region of 3' LTR; WPRE: wood-chuck hepatitis virus posttranscriptional regulatory element; ABP: liver specific promoter (thyroid hormone-binding globulin promoter sequence, two copies of microglobulin/bibunim enhancer sequence, leader sequence); hFIX cDNA: human clotting factor IX cDNA.

serial dilution of the pooled normal human plasma.

Plasma samples from mice were screened for the presence of antibody against hFIX, using an ELISA as described previously[12]. In brief, plates were coated overnight with affinity-purified hFIX protein diluted to 1 g/L with 0.1 mol/L $NaHCO_3$. Diluted plasma samples (1:64, 1:512) were then applied to these wells in duplicates, and antibody against hFIX was detected with horseradish peroxidase conjugated antimouse immunoglobulin G (IgG; Zymed Laboratories, San Francisco, CA). Titers were estimated from a standard curve derived with serial dilutions of a mouse monoclonal anti-hFIX antibody (Roche Molecular Biochemicals).

The biodistribution and expression of hFIX vectors Tissue samples from murine liver, spleen, kidneys, heart, pancreas, testis, and ovary were frozen in liquid nitrogen and then pulverized using a mortar and pestle followed by placement in digest buffer (SDS, Tris-HCl, Proteinase K) at 55 ºC overnight. DNA was isolated from the supernatant by phenol/chloroform extraction and ethanol precipitation. A little genomic DNA was used for PCR. Total tissue RNA was extracted using Rneasy mini kit (QIAGEN) according to instructions. Approximately 1 mg of total RNA from each sample was subjected to the reverse transcription conditions in the presence or absence of reverse transcriptase (RT) using the 1st strand cDNA synthesis kit for RT-PCR of the resulting sample, 5 mL was amplified in a 50-mL PCR reaction as described above. A pair of β-actin primers were used for internal control.

Pathological analysis and immunohistochemistry Part of the treated mice were sacrificed at d 5 *utero* post injection. The liver was obtained for pathologic analysis by hematoxylin/eosin staining. The liver was fixed in Bouin's fixative overnight, embeded in paraffin, sectioned and stained in hematoxylin/eosin. For immunolabeling procedures, the sections were blocked in 5 % milk and were incubated in diluted (1/500) rabbit antiserum to human factor IX, which employed standard ABC techniques, then incubated with peroxidase-conjugated goat anti-rabbit antibody. After being washed, the slides were covered with DAB substrate and developed at 37 ºC for 10 min.

RESULTS

Viability of fetus following intrahepatic injection of recombinant lentivirus We studied the survival rate after intrahepatic injection at 16-18 d gestation with recombinant lentiviral FAXW. Data were analyzed as the number of surviving pups at birth over the total number of pups injected for each group, described as a percentage rate (Tab 1). There was a decrease in survival with FAXW virus compared with control. The causative factors responsible for the difference remains unknown, but either virion proteins like viral protein R (vpr) and VSV-G, which are known to have potential toxic effects, or concentrated contaminants in the viral preparations are supposed to be the resonable reasons. The gene transfer had no adverse effect on fetal growth and development, and postnatal testing of the treated animals revealed no impairment of growth or developmental injury.

Tab 1. Surival rates after intrahepatic injection of virus in fetus.

Viral vector	Dose/ VG·μL^{-1}	Route	Number of injected	Number of surviving	Survival ratio/%
PBS	10 μL	Liver	11	10	90.9
FAXW	8×10^7	Liver	12	8	66.7
FAXW	8×10^7	Liver	10	8	80.0
FAXW	8×10^7	Liver	13	9	69.2

Expression of hFIX in newborn mice Recombinant virus FAXW was injected into the liver of 16-18 d gestation mouse fetus. The concentrations of human factor IX in the plasma of newborn mice were determined by ELISA at d 5, d 10, d 15, d 20, d 25, and d 30. The results showed that plasma hFIX levels reached a peak of 52 μg/L by d 10, then decreased and stably sustained at 20 μg/L for more than 30 d (Fig 2). The results suggested that the recombinant lentiviral FAXW could be efficiently delivered and expressed in the liver of mouse fetus and offered a promising result for further study.

Detection of anti-hFIX antibodies The generation of anti-hFIX antibody responses were assessed in plasma samples collected from neonatal mice of 5 d, 10 d, 15 d, 20 d, 25 d, and 30 d using ELISA. There was no evidence of antibodies generated to hFIX in any of the animals assessed after *in utero* injection.

PCR, RT-PCR analysis of mouse tissues To analyze the distribution and persistence of transduced cells over time, animals were necropsied at birth and samples from all collected tissues were processed for

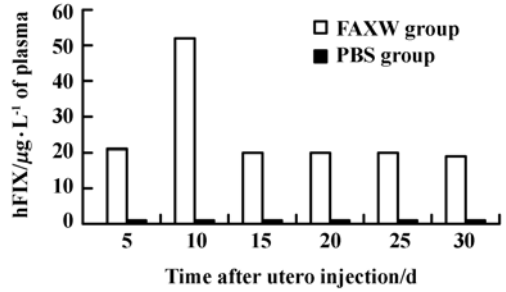

Fig 2. The expression of hFIX followed by *utero* injection with FAXW.

PCR. PCR results indicated that hFIX transgene could be detected in the spleen, liver, and heart. The gonads and the maternal uterus showed negative results by PCR (Fig 3). Biodistribution studies confirmed that most of the gene transfer was limited to the liver, spleen, and heart after *in utero* intrahepatic injection. The transcript of hFIX cDNA was only detected in liver by RT-PCR (Fig 4). A possible explanation for this phenomenon might be that the recombinant FAXW was driven by liver-specific promoter ABP.

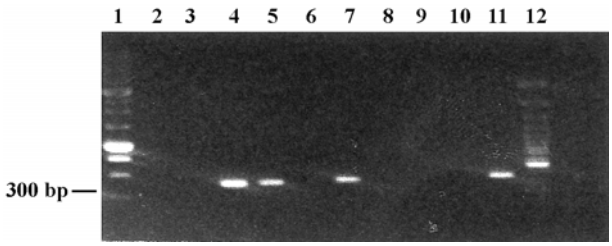

Fig 3. Detection of hFIX cDNA by PCR. Lane 2: testis; Lane 3: ovary; Lane 4: liver; Lane 5: heart; Lane 6: pancreas; Lane 7: spleen; Lane 8: kidney; Lane 9: uterus; Lane10: negative control; Lane11: positive control; Lane 1 and 12: marker.

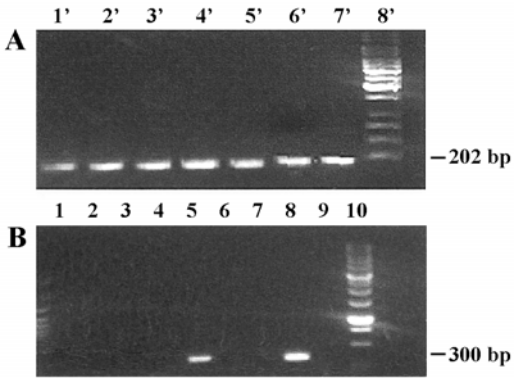

Fig 4. Detection of hFIX cDNA by RT-PCR. A) Eletrophoresis analysis of β-actin RT-PCR products. Lane 1': gonad; Lane 2': ovary; Lane 3': spleen; Lane 4': kidney; Lane 5': liver; Lane 6': heart; Lane 7': materal uterus; Lane 8': marker. B) Eletrophoresis analysis of hFIX RT-PCR products. Lane 1: testis; Lane 2: ovary; Lane 3: spleen; Lane 4: kidney; Lane 5: liver; Lane 6: heart; Lane 7: pancreas; Lane 8: materal uterus; Lane 9: negative control; Lane 10: marker.

Immunohistochemical and pathological analysis Immunohistochemical assessments were carried out on sections of all collected liver samples. In this study, hFIX-positive cells could be observed on sections of liver (Fig 5). No pathological change was observed in major tissues like liver (Fig 6), kidney, or testis, *etc*.

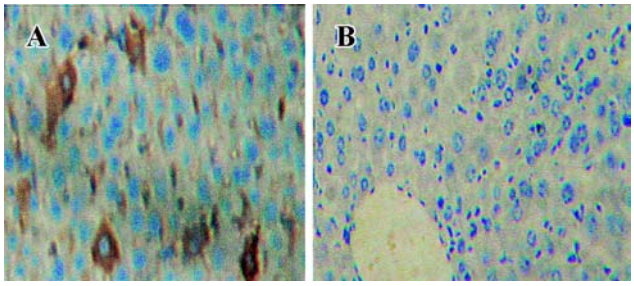

Fig 5. Immunohistochemistry staining for hFIX in liver 5 d after *utero* injection (×100). A) hFIX immunohistochemical staining of live cells; B) Negative control.

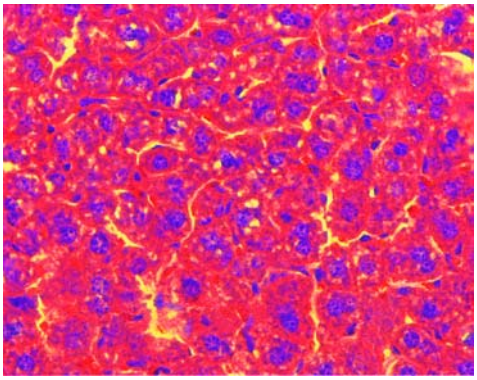

Fig 6. Pathological analysis of liver from neonatal mouse 5 d after *utero* injection (×100).

DISCUSSION

Advances in prenatal screening and molecular diagnosis, combined with the impetus from the human genome project, make it likely that in the near future most genetic diseases will be diagnosed early in gestation. The prenatal diagnosis of genetic diseases will allow increasing opportunities to consider *in utero* gene therapy, particularly if advantages of prenatal treatment can be demonstrated over existing postnatal therapies.

Previous study showed that 350 μg/L of hFIX was

achieved in mice partially hepatectomized by administration of HIV vector[13], the finding demonstrated that hepatocellular proliferation significantly enhanced lentiviral expression of hFIX from mouse liver mediated by lentiviral vector, but the disadvantage made gene transfer more complicated and raised potential risks to donors, which limited this strategy to be popularized and applied in clinic. The intensely proliferative state of the fetus may be in favor of lentiviral vector transduction, as has been shown in animal models of hepatocellular proliferation. In this study, the expression level of hFIX was 52 μg/L and lasted for more than 30 d post injection of FAXW in mice fetus , the reason for low level of hFIX might be due to low dose of virus we used for the treatment. So further improvement in lentiviral vectors will be required to achieve therapeutic levels of hFIX at a dose that is considered to be safe.

For many of the genetic disorders, there is no protein expressed in patients. Thus, transgene expression of therapeutic proteins may elicit immune responses limiting the clinical efficacy. Immune responses limit the efficacy of gene therapy for hemophilias A and B, where inhibitory antibodies restrict the effectiveness of recombinant products[14]. Tsui *et al* showed that high-titer of anti-hFIX antibodies were detected in the serum of the LV-hFIX- transduced C57BL/6 mice, and the kinetics of the appearance of these antibodies corresponded with the loss of serum hFIX expression[13]. In our study, antibodies that reacted with hFIX were not found. A possible explanation for this phenomenon may be the low expression level of hFIX and fetal immune immaturity to induce immune tolerance with transgene. Further study of the mechanism of this observation is required.

The tissue distribution of transgene was analyzed by PCR. The results from assessment of postnatal specimens revealed a more restricted distribution of transduced tissues such as the liver, spleen and heart. The gonads showed negative results by PCR, suggested that the germ cells were not affected. The serious concern for *in utero* gene transfer is the safety of the mother. In this study , we looked for transmission of the transgene across the placental barrier, no transgene was observed in any of the animals studied. These studies demonstrated that direct injection of a recombinant lentivirus *in utero* is a feasible means of safely delivering a foreign gene to a developing fetus and achieving long-term expression without effecting the germ line of the recipient.

To this end, we have developed a system of utero fetal gene therapy for hemophilia B mediated by recombinant lentiviral vector. We firstly demonstrated the feasibility of utero fetal gene therapy for hemophilia B via recombinant lentiviral vector. This novel gene therapy strategy is suitable not only for hemophilia B, but also for other genetic diseases. At the same time, it provides a rapid and simple method for evaluating the expression of foreign gene *in utero* as well as correlative development issues.

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