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Construction of a novel lentiviral vector carrying human B-domain-deleted factor VIII gene

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Vectors derived from human immunodeficiency virus-1 (HIV-1) are highly efficient vehicles for gene delivery. The present study aimed to establish a potent expression system of human factor VIII (FVIII) with lentiviral vectors. FVIII^{BD} gene was obtained by enzyme digestion and inserted into lentiviral vector pXZ208 driven by cytomegalovirus (CMV) promoter/enhancer. Recombinant viral particles were prepared by cotransfection of 293T cells with packaging plasmids NRF and VSV-G through calcium phosphate precipitation. A variety of cell lines including 293T, HLF, NIH3T3, BMEC, Chang-Liver cells and MSCs were infected with recombinant virus containing FVIII^{BD}. The expression of FVIII^{BD} mRNA, FVIII procoagulant activity and genomic DNA integration were detected. All the above cell lines were successfully transfected by recombinant lentiviruses. The transfection efficiencies in 293T, HLF, NIH3T3, mBMEC, Chang-Liver cells and MSCs were 59.57 ± 5.24, 74.52 ± 7.57, 41.33 ± 5.82, 42.34 ± 5.84, 14.38 ± 2.73% and 27.24 ± 6.53, respectively. All the cell lines expressed FVIII after infection to different extents and the activity of FVIII in 293T, HLF, NIH3T3, mBMEC, Chang-Liver cells and MSCs was 43.2 ± 3.2, 54.1 ± 5.6, 14.2 ± 2.8, 8.7 ± 1.3, 22.5 ± 2.9 and 12.5 ± 2.7%, respectively. In addition, FVIII^{BD} mRNA and genomic DNA integration were detected in all cell lines after transfection. A novel lentiviral vector carrying human FVIII^{BD} was constructed, which was able to transfect different mammalian cell types accompanied by high-level activity. This lentiviral vector may provide a theoretical basis for the gene therapy of patients with hemophilia A.

Key words: Lentiviral vector, coagulation factor VIII, transfection, in vitro, gene therapy, prophylactically (Hilgartner, 2002). Purified FVIII concentrates concentrated from human peripheral blood or produced by recombinant DNA and genetic engineering technology are now available for replacement therapy and clinical effectiveness has been achieved (White et al., 1988). However, the replacement therapy has been demonstrated to have some complications. The common and severe adverse effects include: (a) Production of antibodies against exogenous FVIII; (b) contamination by hepatitis B and C; (c) risk of HIV infection (Jones, 1991; Ragni, 1998; Hoyer, 1995; Eyster et al., 1992).

Recent advances in molecular biology and genetic engineering have made gene replacement therapy possible for patients with hemophilia A. Although major progress has been achieved in the replacement therapy of hemophilia A using various gene delivery systems, critical limitations remain the major concerns (Roth et al., 2001; Couzin and Kaiser, 2005; Rawle et al., 2004; Arruda et al.,...
HIV-1-based lentiviral vector, and detect the expression study aimed to investigate the transduction efficiency of numerous investigators due to their ability to transfect non-dividing cells and low immunogenicity. The present study aimed to investigate the transduction efficiency of HIV-1-based lentiviral vector, and detect the expression of target gene in a variety of cell lines transfected with lentiviral vector in vitro.

MATERIALS AND METHODS

Construction of the lentiviral expression vector

Both self-inactivating (SIN) lentiviral vectors pXZ171 and pXZ208 driven by CMV promoter were used in this study. pXZ171 carrying target gene, the enhanced green fluorescent protein (GFP) served as the control, of which the vector was previously constructed by our laboratory (unpublished data). An XhoI fragment containing human B-domain-deleted coagulation factor FVIII (FVIII-BD) gene was excised from pDLZ6, which was kindly provided by Dr. Hengjun Chao (University of North Carolina at Chapel Hill, USA) (Chao et al., 2000), and inserted into the XhoI site of pXZ208 to construct lentiviral expression vector pXZ208-FVIII-BD. The sequence orientation was verified by XhoI and KpnI restriction digestion analysis. The packaging plasmid pMD.G, encoding vesicular stomatitis virus G (VSV-G) envelope protein and the plasmid pCMV ΔBD were previously described (Zufferey et al., 1997).

Cell lines

Chang-liver cells (kindly provided by Dr. Li Xuexia, Xuzhou Medical College), brain microvascular endothelia cells (BMEC) (Dr. Ding Daofang, Shanghai Jiaotong University School of Medicine), 293T and NIH3T3 cells (brought from ATCC) were maintained in Dulbecco’s modified Eagle medium in humidified air with 5% CO₂. HLF cells were grown in modified RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS) in humidified air containing 5% CO₂. Human bone marrow mesenchymal stem cells (MSCs) were isolated and purified as previously described (Krampera et al., 2003; Van Damme et al., 2006).

Preparation of lentiviral vectors

Viral particles were generated by cotransfection of 293T cells with packaging mix as previously described with minor modifications (Naldini et al., 1996; Chen and Okayama, 1997). 293T cells were seeded in 10-cm plates at a density of 2×10⁶ cells/ml 24 h prior to transfection. The medium was changed with that free of serum and antibiotics 3 h before transfection. A total of 30 μg of vectors, including 10 μg of pXZ208-FVIII-BD, 15 μg of pCMV ΔBD, 8.91 and 5 μg of pMD.G, were suspended in a solution containing 125 μl of 1 M CaCl₂, 375 μl of sterilized water, and 500 μl of 2x borate buffered saline, which was incubated at room temperature for 20 min, followed by transferring into the plates. FCS was supplemented at the final concentration of 10%. The medium containing lentiviral particles was collected 48 h after transfection, sterilized through filtration with 0.22 μm cellulose acetate filter and then stored at -80°C.

Determination of viral titers

To determine viral titers, 293T cells were seeded at 2 × 10⁵ cells per well in 6-well plates and maintained for 6 h. The supernatant was replaced with 1 ml of diluted or undiluted vector supernatant containing pXZ171-GFP, followed by incubation overnight. Then, the supernatant was replaced with fresh medium. 48h after lentiviral infection, the cells were observed under fluorescent microscope and the percentage of GFP positive cells was counted. Titers were calculated using the following formula:

\[ \text{Titters (infection unit/ml, IU/ml) = GFP positive cells} \times \text{dilution multiple} \]


Cell transfection

Cells were seeded at a density of 1 × 10⁶/well. 24 h later, the medium was removed and replaced with 5 ml of medium containing 8 μg/ml polybrene and viral vectors. 48 h later, the GFP-positive cells were counted under fluorescent microscope and analyzed by flow cytometry (FCM).

Assay of coagulation factor activity

The supernatant of infected cells was collected for FVIII activity assay with one-stage method (Factor Deficient Plasmas, Pacific Hemostasis, USA) 48 h after transfection.

Detection of FVIII-BD mRNA expression

Total RNA extracted from cultured cells were used for reverse transcription (RT)-PCR (RNAgents® Total RNA Isolation System & Access RT-PCR System, Promega). The primers and reaction conditions were described elsewhere (Chao et al., 2000). Briefly, the forward primer 5'-TTCTCCCCAATCCAGCTGG-3', and the reverse primer 5'-GAGTTATTTCCCGTGATGG -3' were used to amplify a 534 bp fragment. Reverse transcription contained denaturation at 42°C for 10 min and termination reaction at 95°C for 5 min. The cycling conditions for PCR were 30 cycles of denaturation (95°C for 2 min), annealing (55°C for 1 min) and extension (72°C for 1 min). A preheating step at 95°C for 5 min and a final extension step consisting of 4 min at 72°C were also carried out.

Detection of DNA integration of FVIII-BD

To verify whether the FVIII-BD was stably integrated into the genomic DNA, high molecular weight DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions. PCR-based amplification of the target fragment was performed and the cycling conditions were prede-

Statistical analysis

Data are presented as mean ± SD. The means of transduction efficiencies and FVIII secretion levels of cells transduced were...
Figure 1. Restriction enzyme map of recombinant plasmid pXZ208-BDDh FVIII. M: 1,000 bp DNA Ladder; 1: pXZ208-FVIII³BD (Xho I); 2: pXZ208- FVIII³BD (Kpn I); 3:pXZ208 (XhoI); 4:pXZ208 (KpnI).

compared with the use of a 2-tailed paired student’s t test. Statistical analysis was performed using the GraphPad Prism 3.0 software.

RESULTS

Verification of transfer vector

The recombinant FVIII³BD fragment was inserted into the downstream of CMV promoter to construct the recombinant vector pXZ208-FVIII³BD. The full length of pXZ208-FVIII³BD was 13,018 bp. After digestion with XhoI, two fragments, 4435 and 8583 bp were obtained (Figure 1, lane 1). Digestion with KpnI resulted in fragments of 3341 and 9677 bp (Figure 1, lane 2). pXZ208 was also excised with XhoI or KpnI as blank vector control (Figure 1, lanes 3 and 4).

Detection of transfection efficiency of the recombinant vector GFP expression by fluorescent microscopy

The viral titers were as high as 2.18 ± 0.64 × 10⁶ IU/ml, which could be further concentrated to 10⁹ IU/ml. Cells were exposed to pXZ171-GFP at a concentration of 10⁶ IU/ml for 48 h. The expression of GFP varied among different cell lines. GFP was highly expressed in 293T and HLF cells, whereas the green fluorescent signals were relatively weak in BMEC cells (Figure 2). The number of GFP positive cells in 293T and HLF cells was markedly higher than that in BMECs and MSCs (Figure 2). These findings were further confirmed by FCM analysis (Table 1). The transfection efficiencies of different cell types were compared and the results showed that the transfection efficiency was highest (P < 0.05) in HLF cells, while the lowest transfection efficiency was noted in BMECs (P < 0.05) (Figure 3).

Detection of FVIII³BD mRNA and protein activity in different cell types

293T, HLF, NIH3T3, BMEC, Chang-Liver cells and MSCs were transfected with lentiviral vectors. The results showed that therapeutic factor VIII activity levels were observed in all cell types transfected with lentiviral vectors (Table 2). RT-PCR was performed to verify the mRNA expression of FVIII³BD. The results demonstrated that the fragment of target gene (534 bp) was amplified in all cell lines (Figure 4).

DNA integration of FVIII³BD

Primers were designed for the detection of specific FVIII³BD gene fragment. A specific fragment of 534 bp spanning the genomic integration site and FVIII³BD fragment were amplified in all cell types with lentiviral vector transfection. No target fragment was observed in control cells without target gene transfection (Figure 5).

DISCUSSION

HIV-based lentiviral vectors are highly efficient gene delivery vehicles. However, one major concern in clinical trials is its biosafety. In earlier reports, viral particles were generated to simultaneously express viral core enzymes and other capsid proteins. This method gives rise to replication competent lentiviruses (RCLs). To make the HIV-based vectors safer, a major modification of HIV-based lentiviral vector was made in the present study. Promoter and enhancer elements of the U₃ region of long terminal repeat (LTR) were removed in our vector series. The 3’ LTR-directed transcription was eliminated in these vectors resulting in SIN of these vectors following transfection. The use of VSV-G as virus envelope confers stability to the particle and facilitates particle concentration during collection (Burns et al., 1993). Viral particles are traditionally produced by transient cotransfection of 293T packaging cells with three or four different plasmids encoding pol II core enzymes and capsid proteins. The absence of overlapping sequences between plasmids minimizes the possibility of generation of RCL (Aiken, 1997; Parolin et al., 1994). Up to date, no RCLs have been generated from SIN vectors when they were used as gene delivery vehicles in vivo (Naldini et al., 1996; Zufferey et al., 1997; Miyoshi et al., 1997; Goldman et al., 1997; Levine et a., 2006). Furthermore, because no viral
proteins are expressed, these vector systems will not trigger an immune response against cells expressing vector proteins in vivo.

In the present study, virus titers were as high as 2.18 ± 0.64 × 10^6 IU/ml. Similar to previous studies, we have used human HIV-1-based lentiviral vectors for stable gene transfer because it was found that these vectors could efficiently transduce a variety of nondoning cells in vivo (Naldini et al., 1996; Naldini et al., 1996; Miyoshi et al., 1997; Kafri et al., 1997). Although relatively high transfection efficiency was observed in all cell lines, the transduction efficiency varied profoundly in different cell types. The transductions resulted in different levels of FVIII secretion. FVIII:C levels corresponded to different virus titers per 48 h and were among the highest levels reported for FVIII so far in other recombinant systems. Therapeutic levels of FVIII secretion in 293T and HLF transduced with the same vector but 10- to 20-fold lower levels in MSCs and BMECs that were transduced with the same transgene cassette could also be achieved. More GFP-positive cells in 293T and HLF were observed compared to those in NIH3T3, BMECs, Chang-Liver cells and MSCs. This may be attributed to, at least in part, the activation status and cell cycle phases. In addition, cell surface receptor for virus may also be responsible for various transfection efficiencies (Grant and David, 2004). Transfection efficiency in MSCs was up to 5% which has been reported in the detection using electroporation (Matthews et al., 1993). The present studies suggested

Table 1. Transfection efficiency 48 h after infection.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>n</th>
<th>Transfection efficiency (%)</th>
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<tbody>
<tr>
<td>293T</td>
<td>7</td>
<td>59.57±5.24</td>
</tr>
<tr>
<td>HLF</td>
<td>5</td>
<td>74.52±7.57</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>4</td>
<td>41.33±5.82</td>
</tr>
<tr>
<td>MSCs</td>
<td>4</td>
<td>42.34±5.84</td>
</tr>
<tr>
<td>Chang-liver</td>
<td>5</td>
<td>27.24±6.53</td>
</tr>
<tr>
<td>BMECs</td>
<td>6</td>
<td>14.38±2.73</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. 293T, MSCs, BMECs, Chang-liver cells and NIH3T3; *P < 0.05 vs. NIH3T3, 293T, MSCs, HLF and Chang-liver cells.

Figure 2. GFP expression in target cells 48 h after transfection (×100). A, B, C, D, E and F were representative fluorescent photographs of 293T, HLF, NIH3T3, BMEC, Chang-liver cells and MSCs, respectively, under fluorescent microscope.
Table 2. Activity of FVIII in different cell types 48 h after transfection.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>n</th>
<th>APTT (s)</th>
<th>FVIII activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>293T</td>
<td>7</td>
<td>68.5 ± 4.1</td>
<td>43.2 ± 3.2</td>
</tr>
<tr>
<td>HLF</td>
<td>6</td>
<td>63.1 ± 6.1</td>
<td>54.1 ± 5.6*</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>6</td>
<td>93.1 ± 5.7</td>
<td>14.2 ± 2.8</td>
</tr>
<tr>
<td>MSCs</td>
<td>6</td>
<td>97.4 ± 7.4</td>
<td>12.5 ± 2.7</td>
</tr>
<tr>
<td>Chang-liver cells</td>
<td>6</td>
<td>81.5 ± 6.3</td>
<td>22.5 ± 2.9</td>
</tr>
<tr>
<td>BMECs</td>
<td>6</td>
<td>102.6 ± 4.8</td>
<td>8.7 ± 1.3*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. 293T, MSCs, BMECs, Chang-liver cells and NIH3T3; *P <0.05 vs. NIH3T3, 293T, MSCs, HLF and Chang-Liver cells; APTT, activated partial thromboplastin time.

Figure 3. Percentage of GFP positive cells detected by FCM.

Figure 4. Expression of FVIII::BD mRNA detected by RT-PCR. A. Lane 1, 3, 5 were 293T, NIH3T3, and HLF cells, respectively; Lane 2, 4, 6 were controls; B. Lane 1, 3, 5 were MSCs, BMECs and Chang-liver cells, respectively; Lane 2, 4, 6 were controls.

that higher transduction efficiency could be achieved in MSCs, resulting in higher FVIII activity in vitro. Ideal gene therapy aimed to integrate the target gene into the host genome with long-term expression. In the present study,
PCR confirmed gene integration in all cell lines with transfection (Zhang et al., 2002).

Conclusion

The present study provided a basis for further investigating HIV-1-based lentiviral vectors in gene therapy of patients with hemophilia A ex vivo. But it raised several issues. Constant efforts should be made toward the standardization in isolation and purification of MSCs. Some investigators have demonstrated that viral injury causes normally quiescent cells to progress into the cell cycle to enhance lentiviral transduction. For this reason, to produce therapeutic levels of coagulation factors, techniques need to be developed to promote cell cycle progression in a safer, nonsurgical manner, such as the use of growth factors (Bosch et al., 1996; Patijn et al., 1998).

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