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An engineered EBV vector expressing human factor VIII and von Willebrand factor in cultured B-cells

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Received: 14 August 2003
Revised: 19 December 2003
Accepted: 2 January 2004

Abstract

Background Hemophilia A is a congenital disorder caused by a deficiency of the blood-clotting factor VIII (FVIII) and is an attractive candidate for gene therapy. Most of the studies have only explored the potential of hepatocytes and muscle cells as the targets for gene transfer. Attempts to transfer the genes into hematopoietic cells have so far been mostly unsuccessful due to inefficiency of most viral vectors to transduce these cells and the supposed inability of the cells to express FVIII.

Methods We demonstrate the generation of an engineered Epstein-Barr virus (EBV) vector with a BAC backbone that has the unique capacity to carry either the full-length FVIII cDNA or its B-domain-deleted form; a modified version of the vector that carries B-domain-deleted FVIII along with the von Willebrand factor (vWF) cDNA or the reporter gene DsRed2 was also used. All these vectors have been safely modified with viral thymidine kinase cDNA to transduce human B-cells in culture.

Results Low-level expression of FVIII in the order of 5–8 ng FVIIIC/ml were observed in the cells stably transduced with full-length FVIII, while cells with the B-domain-deleted version expressed 8–16 ng FVIIIC/ml. Expression of vWF and B-domain-deleted FVIII resulted in a moderate expression of 18–30 ng FVIIIC/ml. Long-term expression for 12–16 weeks was observed in these cells regardless of selection pressure.

Conclusions These results support the development of an episomal engineered EBV vector for treatment of hemophilia A using the hematopoietic cells as a target for providing immediate secretion of functionally active product in the circulating bloodstream. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords EBV; hemophilia A; von Willebrand factor; hematopoietic cells; gene therapy

Introduction

Hemophilia A is an X-chromosome-linked severe bleeding disorder affecting about 1 in 5000–10,000 males worldwide. Clinically, the disease is characterized by episodes of spontaneous bleeding primarily in soft tissues and joints, which in turn render the patient disabled. Moreover, in the most severe cases, it can cause life-threatening hemorrhages in the central nervous system. The disease is caused by a deficiency in coagulation factor VIII (FVIII). FVIII is an essential component of the intrinsic pathway of the blood coagulation cascade, where it serves as a cofactor for activated factor IX (1–3).
Based on the residual activity of FVIII in peripheral blood plasma, hemophilia A is categorized as mild (5–30% of normal activity), moderate (1–5%) and severe (<1%). Current prophylactic treatment of the disease is based on intravenous infusion of plasma-derived or recombinant FVIII. The major disadvantages of such replacement therapy include not only the inconvenience of frequent injection and expense, but also the risk of transmission of viral-borne diseases such as HIV and hepatitis. These shortcomings of replacement therapy, however, make hemophilia A an attractive candidate for gene therapy.

The liver, which is the natural site of production of FVIII, is the most preferred target tissue for hemophilia A. However, recombinant FVIII expression has been shown by a variety of cells including epithelial as well as endothelial and myoblasts [4]. Furthermore, the severity of the disease can be dramatically decreased with even modest elevation (5–10% of normal) of FVIII levels in the plasma. Consequently, it has been suggested that precise and tissue-specific regulation of FVIII expression may not be of critical importance. Due to limitation of retroviral vectors to transduce non-profiferating cells of the liver, extra hepatic tissues are being explored as targets for hemophilia A gene therapy. Recently, bone marrow derived stromal cells have also been investigated as a target for gene therapy of hemophilia [5]. An alternative attractive target would be the hematopoietic cells themselves due to their immediate accessibility to the circulating bloodstream. However, except for a very few studies, attempts to express recombinant FVIII in hematopoietic cells have so far been unsuccessful [6–8]. We have therefore used a second-generation infectious, episomal EBV-based vector to transduce human B-cells in culture. Previously, the natural tropism of EBV has been exploited to deliver cDNAs into human B-cells using a mini-EBV vector [9,10]. In the present study, we have incorporated the BAC backbone in the EBV-based vector, so that the large insert can be cloned and propagated in bacteria as well as shuttled into mammalian cells.

Recently, a similar EBV ampiclon vector has been used to deliver genomic DNA of 120 kb into a human B-cell line [11]. The engineered EBV used in this study has the viral thymidine kinase (tk) gene as a safety switch; the vector carries either the full-length or the B-domain-deleted FVIII cDNA. In a further modification of the vector, both the B-domain-deleted factor VIII cDNA and the vWF cDNA were incorporated as the therapeutic genes. Using this EBV vector system and B-lymphoblastoid cells we were able to show secretion of functionally active factor VIII in the surrounding media.

Materials and methods

Construction of B-domain-deleted FVIII cDNA

The full-length FVIII cDNA (ATCC Accession No. 40086) was cloned using linker tailing into the NotI site of a modified pREP7 vector (Invitrogen, USA) deleted of other multiple cloning sites to obtain the plasmid pRBl. Using PCR, a B-domain-deleted FVIII cDNA (4.6 kb), more commonly used in gene therapy research, was constructed. To delete the major portion of the B-domain from the full-length FVIII cDNA, i.e. nucleotides ranging from 2504 to 5144, PCR amplification of the upstream 5’-region of the B-domain was performed with the primer pair 5'-GAACCGAAGCTGGTACCT-3’ and 5'-GACAGGAGGGCATTAATGCTTTTGCCCT-3’ and downstream 3’-region of the B-domain with 5’-TTTAAATGGCCACCCAGCTCTTGAAACGCCAT-3’ and 5’-ATGCTGCCAATAAGGCCATTCGGA-3’ using high fidelity polymerase (Becton Dickinson Biosciences-Clontech, USA). After amplification, these two products were heat-denatured followed by renaturation and then extended to yield the desired deletion. The KpnI and BglII digested amplified product was then cloned into KpnI-BglII digested full-length FVIII cDNA carrying plasmid after removal of its own fragment. We thus sub-cloned the B-domain-deleted FVIII cDNA (BDFVIII) under the control of the RSV 3’LTR in pREP7 (Invitrogen, USA) to obtain pRBD8F.

Using primers specific for different exons, PCR of the BDFVIII construct confirmed the absence of the B-domain (data not shown).

Construction of vector

The EBV-BAC vector carrying the FVIII cDNA and the von Willebrand factor cDNA was constructed in several steps. The latent origin of replication oriP and the selection marker hyg1 was excised out of the vector pH200 [12] using Sall. This Sall-digested fragment of oriP-hyg1 was then ligated to the Xhol site of pBlol Bac11 [13]. Using the blunt end site Hpal in this modified BAC vector, the EBNA1 gene from the pCMV EBNA1 vector (Invitrogen) was retrofitted to obtain pBl. The HSV thymidine kinase (tk) gene [14] under the control of the SV40 viral promoter was sub-cloned into the unique HindIII site of the oriP-hyg1-deleted pH200 vector to obtain pE1. The Sall-digested expression cassette for the full-length FVIII cDNA from the plasmid pR8F or for the B-domain-deleted FVIII cDNA from the plasmid pRBD8F was then ligated by partial fill-in to the unique BamHI site [9] of the modified pE1 to obtain pEF8 or pEBDFS. The resultant plasmid was then redigested with Sall and retrofitted into the Sall site of pBl1 to finally obtain pEBF8 or pEBDFS.

A modified version of this EBV-BAC series which incorporates both BDF8 and vWF cDNA was also constructed. This was a low copy vector deleted of the pBR322 replication origin. The plasmid pEBDFS12 was digested with Fsp1 and the blunt end fragment ligated into the blunt end Sal I filled-in site of pBl1 to obtain pEB-BDF8. The vWF cDNA (ATCC Accession No. 591250) was initially cloned into the NotI site of pCE4 under the control of the cytomegalovirus early (CMV) promoter and the Sall-digested expression cassette was retrofitted.

EBV for Gene Therapy

Production of recombinant EBV was performed as previously described [9,10]. However, B95-8, an EBV-positive cell line obtained from the National Centre for Cell Sciences (Pune, India), was used as a source to produce the recombinant virus. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μg of penicillin/ml, 100 μg of streptomycin/ml and 50 μg of neomycin/ml (GIBCO-BRL, USA). Plasmids pEBFP8, pEBBD8, pEB-BDF8/vWF, and pEB-BDF8/DsRed2 were separately electroporated into B95-8 cells and the transfected cells were then selected in the presence of 200 μg/ml of hygromycin B. Hygromycin-resistant B95-8 cells were then used to produce recombinant EBV by treating the cells with TPA and sodium butyrate. The induced medium was then filtered through 0.45-μm cellulose acetate filters and the filtrate was then used for infection of B-lymphoblastoid cells.

Infection of hematopoietic B-cells with recombinant EBV

Raji, a human B-lymphoblastoid EBV-positive cell line, was obtained from the National Centre for Cell Sciences (Pune, India). DG-75, BJAB, and BL-41 are human EBV-negative B-cell lines kindly provided by Dr. A. Rickinson (UK) and Dr. G. Kline (Sweden). Briefly, the cells (5 × 10⁶) were washed in phosphate-buffered saline (PBS), mixed with 0.5 ml of concentrated virus (or filtrate) and incubated at 37°C for 2–3 h with gentle shaking, centrifuged, washed, and plated as described previously [9,10]. In order to evaluate the efficiency of transduction, engineered viornes carrying the reporter gene DsRed2 (BD-Clonetech, USA) cloned into pCEP4 were sub-cloned using the SalI digestion. The detailed flow chart and method of construction of the vector are available on request.

Stable cell transformants, however, were selected in the presence of 200 μg/ml of hygromycin B (GIBCO-BRL).

Episomal maintenance of the vector

Episomal DNA was extracted from stably transduced cells as described previously [9,10,12]. In brief, after washing with PBS, 5 × 10⁷ cells were suspended in 5 ml of lysis solution (50 mM NaCl, 8 mM EDTA, 1% SDS; pH 12.45) and incubated at 37°C for 30 min. Then, 1 ml of Tris (pH 7.0), 660 μl of 5 M NaCl and 660 μl of proteinase K (10 mg/ml) were added with gentle mixing, incubated at 37°C for 30 min, followed by standing at room temperature for 5 min. Phenol (4 ml; saturated with 0.2 M NaCl, 0.2 M Tris-HCl) was then mixed very gently and centrifuged. The aqueous phase was then extracted twice with phenol before and once with 5 ml of CHCl₃. From the aqueous phase episomal DNA was precipitated with addition of 11 ml of cold 100% ethanol.

The epimoses were then separated on 0.85% agarose gel and transferred to a nylon membrane (Bio-Rad, USA) and hybridized with vector and EBV-specific probes, labelled pBeloBAC11 and EBNA-2, respectively, using the Random priming labeling kit (GIBCO-BRL) and detected by autoradiography.

RNA isolation and analysis of FVIII expression by RT-PCR

Total RNA was isolated from the stably transduced B-cells with the RNAqeuous™ kit (Ambion, USA). DNA-free RNA (10 ng) was subjected to RT-PCR using the Titan One Tube RT-PCR system (Roche, USA). The transcriptional expression of full-length and B-domain-deleted FVIII cDNA was detected with primer pair #1 (5'-AGAACAAAGAAACACACAGCTG-3') and #2 (5'-TCCGTAGGGAATAGGTCC-3') and #3 (5'-CTCGTAGGGAATAGGTCC-3') and #2 (5'-TGATGAGAACCGAAGCTGG-3' and 5'-GTCAAACTCATCTTAGTGGGTGC-3'), respectively. RT-PCR conditions were: initial incubation at 50°C for 30 min for first-strand cDNA synthesis, then 10 min denaturation at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at annealing temperature and 2 min at 68°C, and final extension at 68°C for 5 min. Control experiments were performed with RNA isolated from cells mock-infected with virus of B95-8 cells stably transfected with vector without the FVIII transgene. The annealing temperatures were 49 and 59°C for full-length and B-domain-deleted FVIII, respectively.

Immunostaining of cells expressing FVIII

Cells were immunostained with anti-FVIII-monoclonal antibody CLB-Cag A (gift from Dr. J van Mourik, The Netherlands) [15]. Cell suspension (10 ml) was concentrated to 100 μl and a smear was drawn on the slide. Cells were fixed in methanol at −20°C for 5 min, and...
EBV for Gene Therapy

transduced B-cells. The EBV plasmid is in essence a derivative of the first-generation mini-EBV plasmid pH200. It contains essentially the latent and lytic replication origin oriP and ori lyt, respectively, the packaging signal TR, the hygromycin-resistance gene and additionally contains a BeLOBAC11 backbone along with the EBNA1 transactivator and the thymidine kinase gene. The RSV long terminal repeat (LTR) was used to express the FVIII cDNA in this vector (Figure 1A). The modified vector carrying both B-domain-deleted FVIII and the vWF cDNA (or DsRed2), however, was a low copy plasmid as it was deleted of the pBR322 replication origin and only contained the BeLOBAC 11 backbone (Figure 1B). The transduction of B-cells was visualized and photographed (1000x magnification) for DsRed2 expression under a fluorescence microscope (Figure 2). Our cytometric data indicate the transduction efficiency after 72 h of infection for Raji, DG-75, BJAB and BL-41 is approximately 35, 30, 20 and 25%, respectively.

Southern blotting with pBeLOBac11- and EBV-specific probes for stably transduced cells either carrying the full-length FVIII cDNA or the B-domain-deleted version grown on selection with hygromycin for about 90 days confirmed the maintenance of episomal DNA as a closed circular DNA of 170 kb, the approximate size of the supercoiled EBV genome (Figure 3). This is in agreement with previously reported data [9,10] and confirms that the EBV-based vector is episomal in B-lymphoblastoid cells.

In vitro expression of FVIII and vWF in transduced cells

To detect transcription of vector-encoded FVIII cDNA, RT-PCR was performed with total RNA isolated from cells

Figure 2. Fluorescent microscopic photograph of DG-75 cells transduced with pEB-BDF8/DsRed2 (1000x). The percentage of DsRed2-positive cells was determined by flow cytometry

Figure 3. Southern gel analysis for episomal maintenance of pEBF8 and pEBD8 (lanes 1 and 2) in stably transduced Raji cells in selection for 90 days and probed with pBelOBAC11 or EBNA2. The closed circular episomal EBV (170 kb) marker is indicated on selection with hygromycin for about 90 days confirmed the maintenance of episomal DNA as a closed circular DNA of 170 kb, the approximate size of the supercoiled EBV genome (Figure 3). This is in agreement with previously reported data [9,10] and confirms that the EBV-based vector is episomal in B-lymphoblastoid cells.

In vitro expression of FVIII and vWF in transduced cells

To detect transcription of vector-encoded FVIII cDNA, RT-PCR was performed with total RNA isolated from cells

Figure 5. Immunostaining with anti-FVIII antibody CLB CAg A of Raji B-cells transduced with (A) mock-infected control and (B) B-domain-deleted FVIII virions. Black dots indicate expression of FVIII

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J Gene Med 2004; 6: 000–000.
washed with PBS and then incubated with antibody, CLB-CAg A (1:1000 dilution in PBS), for 2 h in a humidified chamber at room temperature. After washing with PBS, biotin-conjugated anti-mouse secondary antibody (1:1000 dilution in PBS) was overlaid on it and incubated for 30 min. After washing the slides and incubating with NBT/BCIP reagent for 45 min, it was counter-stained with 1% eosine for 5 s, washed with ethanol and observed under 1000× magnification.

Detection of vWF

Expression of co-transduced vWF in the pEB-BDF8/vWF-carrying cells was confirmed by RT-PCR using the Titan One Tube RT-PCR system (Roche), and the primer pair #3 (5'-CAACAAAGGAACGTCTCC-3' and 5'-TACACAACAGAGCCATTGG-3') to amplify a ~758 bp product specific for vWF cDNA. Briefly, the RT-PCR conditions were: initial incubation at 50 °C for 30 min for first-strand cDNA synthesis, then 10 min denaturation at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 59 °C, and 2 min at 68 °C, and final extension at 68 °C for 5 min.

Detection of FVIII activity

Biological activity of FVIII was detected by a chromogenic assay that relies on FVIII-dependent activation of factor X, which in turn produces p-nitroaniline after cleavage of the substrate (COATEST VIII: C/4, Chromogenix, Italy). To construct a standard curve of FVIII activity, blood samples were collected from 20 healthy volunteers, centrifuged at 2000 g for 15 min at room temperature and the supernatants were then pooled and serially diluted. FVIII activity from each diluted set was then determined and plotted against absorbance to generate a standard curve, assuming the normal level of FVIII to be 200 ng/ml in healthy plasma [1–3]. Raji, DG-75, BJAB, and BL 41 cells transduced with pEBF8, pEBBDF8 or pEB-BDF8/vWF were used to detect functional activity of FVIII; mock-infected cells with only vector without the transgene were used as negative controls. Three separate sets of experiments were performed for each type of cells.

ELISA for detection of FVIII in culture medium

First, 96-well plates were coated with 50 μl of monoclonal anti-FVIII antibody, CLB Cag 9 (a gift from Dr. J. van Mourick, The Netherlands; 10 μg/ml), at 4 °C for at least 16 h. Wells were then washed four times with wash buffer (0.9% NaCl, 0.1% Tween 20). Non-specific sites were then blocked with 100 μl of blocking buffer (0.25% BSA in PBS containing 0.1% Tween 20) for 1 h at 37 °C. After washing four times, 50 μl of cell-culture medium or plasma from healthy individuals diluted in blocking buffer without BSA were added to coated wells and then incubated for 2 h at 37 °C. Anti-FVIII antibody CLB Cag A (400 ng/ml; biotinylated) was then added and incubated for 2 h at 37 °C. Wells were then washed four times and 50 μl of 500-times diluted alkaline phosphatase-streptavidin conjugate (Bangalore Genei, India) were added to each well. After 1 h of incubation at 37 °C, each well was washed four times and incubated with 50 μl of 10 mg/ml of p-nitrophenyl phosphate, dissolved in substrate buffer (9.7 ml of diethanolamine, 10.1 mg of MgCl2 in 100 ml of H2O2; pH 9.5) and incubated at 37 °C for 20 min. Then the absorbance was measured at 405 nm. From the standard curve obtained with serially diluted plasma of healthy individuals, FVIII concentration in the culture medium was calculated.

Results

Stable transduction of B-lymphoblastoid cells in culture and episomal maintenance of the vector

Due to the presence of oriP, the latent replication origin of EBV and its transactivator EBNA1, the vector existed as an extrachromosomal element in the...
Secretion and biological activity of FVIII

Many studies have shown that, in general, FVIII is poorly expressed in mammalian cells in culture which is due not only to a low-level steady-state FVIII mRNA, but also to its inefficient processing and secretion into the surrounding media. However, ectopic expression of both full-length and the B-domain-deleted FVIII cDNA could be detected in the supernatant culture media of transduced B-lymphoblastoid Raji, DG 75, BJAB, and BL-41 cells as determined by chromogenic assay as well as a sandwich ELISA. It may be noted that analysis of the supernatants for FVIII secretion was higher for cells transduced with B-domain-deleted cDNA compared with full-length cDNA (Figure 6). Full-length FVIII exhibited a low expression level of 5-8 ng/ml, while B-domain-deleted cDNA exhibited an activity of 8-16 ng/ml. However, infection with vector carrying the von Willebrand factor along with B-domain-deleted FVIII exhibited a marked augmentation in the level of expression of FVIII in the transduced cells. In order to investigate the sustained expression of FVIII, supernatants of transduced cells grown in the presence and absence of any selection were subjected to ELISA (Figure 7). In comparison with cells grown on selection, the activity of FVIII decreases to ~20% for stably transduced Raji cells grown for a period of 90 days in the absence of hygromycin.

Discussion

To summarize, in the present study we have been able to show the efficacy of an EBV vector with a BAC backbone to transfer full-length FVIII cDNA or the B-domain-deleted FVIII cDNA along with the vWF cDNA into human B-lymphoblastoid cells in culture. The ectopic expression of the therapeutic genes was studied by RT-PCR, ELISA and immunostaining of the stably transduced cells. Functional activity of FVIII secreted in culture medium from the cells carrying the vector as confirmed by a chromogenic assay, (COATEST VIII):
Figure 7. Long-term persistence of FVIII activity in absence of selection, in culture supernatant of Raji cells with pEBBDF8. Stably transduced cells (10^9 cells/ml) grown in the absence of hygromycin for different times were used to assay FVIII activity with ELISA.

EBV for Gene Therapy

1. EBV-based replicon plasmid can be transferred into lymphotrophic viral vector has the natural potential to infect cells of the hematopoietic lineage for gene therapy. One can also argue that for cells to escape the immune reaction targeted against the viral vector. In this regard, adenoviral vectors that can transduce a wide variety of cells have also been used for efficient FVIII production. However, the vector sometimes elicits very strong immune responses that lead to gradual loss in expression of the gene. Since EBV is present asymptotically in more than 90% of the adult human population, it has evolved mechanisms to escape human immune surveillance and ensures life-long persistence.

2. Although a 10% increase in FVIII level in our in vitro cell model is rather modest, clinical application of this technology would be through a hematopoietic stem cell transplant. In this manner, the FVIII-expressing stem cells would give rise to mature progeny T-cells, B-cells, neutrophils, macrophages and dendritic cells. Even though EBV has long been considered as B-lymphotropic, recent reports have shown the potency of the virus to infect monocytes/macrophages, neutrophils, T-cells and dendritic cells [17–19]. Therefore, this human lymphotrophic viral vector has the natural potential to infect cells of the hematopoietic lineage for gene therapy. Interestingly, studies in mice have shown that an oriP-EBNA1-based replicon plasmid can be transferred into the liver almost specifically by tail-vein injection using a hydrodynamic method [20,21]. Therefore, the second-generation EBV replicon vector could also be delivered as naked plasmid DNA into liver, the natural target of hemophilia gene therapy. One can also argue that for cells that can be easily transfected, an episomal EBV plasmid vector may be a better option than an infectious episomal virus. However, because of its lymphotrophic nature, transduction efficiency of the infectious engineered virions is much higher than transfection.

3. In order to make gene therapy practicable, what we really need is the life-long persistence of the expression of the therapeutic gene. Retroviral and lentiviral vectors, the most commonly used vectors, ensure this by integration into the host chromosome. However, it might also be disadvantageous as non-specific integration of vector could lead to activation (or silencing) of some cellular genes raising a major safety concern [22]. Recently, the occurrence of T-cell lymphoproliferative disorders in patients undergoing clinical trials for gene therapy of severe combined immunodeficiency has been correlated with the integration of the vector into a protooncogene [25]. In this regard, an episomal vector is potentially safer than its integrative counterparts. Among all the viral vectors, an EBV-based episomal vector has the unique capacity to carry a large insert [24] and the presence of the thymidine kinase gene in this engineered EBV has widened the scope of selective elimination of the vector-carrying cells (data not shown), if required for safety reasons. Recent studies with lentiviral vectors pseudocopied with vesicular stomatitis virus (VSV) G-enveloped protein indicate the feasibility of human B-cells as a target for gene therapy of diseases associated with secretion factors and immune dysfunction [25,26]. In this regard, an EBV-based episomal vector could arguably have an advantage over integrative lentiviral vectors to transduce its natural host, the B-lymphocytes, where the virus persists throughout life.

4. Another major challenge for viral gene therapy is to escape the immune reaction targeted against the viral vector. In this regard, adenoviral vectors that can transduce a wide variety of cells have also been used for efficient FVIII production. However, the vector sometimes elicits very strong immune responses that lead to gradual loss in expression of the gene. Since EBV is present asymptotically in more than 90% of the adult human population, it has evolved mechanisms to escape human immune surveillance and ensures life-long persistence. Therefore, the engineered EBV vector that only encodes the viral protein EBNA1 may escape the host immune system [29]. However, one should also be cautious and consider the role played by the episomal element EBNA1, if any, in EBV-positive malignancies. One study demonstrated the EBNA1-induced expression of bcl-xl, RAG 1 and 2 [30], whereas another study showed EBNA1-mediated downregulation of HER2/neu oncogene [31]. Therefore, if the EBV-BAC transduced cells are neoplastically transformed due to the expression of EBNA1, there is always the possibility of selective elimination of the cells using the tk gene as a safety switch. Although in this study we have used a transforming virus producing helper cell line, the non-transforming HH514 helper cell line could be used instead [9–11]. The EBV-based vector when packaged into infectious viral particles exists as an ~170-kb vector-concatamer [9] and the transduced cells have more than one copy of the therapeutic gene, which essentially increases the effective gene dose. Recently, by deleting the packaging signal (TR) from wild-type EBV, a helper-virus-free cell line has been developed that may lead to further development of EBV as a gene delivery tool [32]. Using the EBV-based episomal plasmid vector, human peripheral blood CD34+ cells have been successfully transfected [33]; it therefore remains to be seen whether EBV-based viral vectors could infect these CD34+ cells or pro-B cells as efficiently as mature B-cells and long-lived memory B-cells. However, with this EBV-BAC system, packaged in a transforming virus-free helper cell line, it is feasible to transduce human hematopoietic...
cells ex vivo followed by infusion in a SCID mouse model to properly explore its therapeutic potential.

To the best of our knowledge, this is the first report to demonstrate the potential of EBV-based episomal viral vectors to transfer and express both FVIII and von Willebrand factor from human B-cells. Expression levels of FVIII in B-cells in the presence of WF expression followed by secretion were shown to be slightly higher compared with other reports [8]. It has been previously reported that simultaneous expression of FVIII and Wf cDNAs in the target cells leads to increased FVIII expression as well as secretion in the surrounding media [1,2]. FVIII serves as a pivotal molecule to carry FVIII and the binding of FVIII with WF leads to stabilization of the FVIII and reduced proteolytic cleavage. Moreover, recent reports suggest that FVIII complexed with WF may be inhibitory for generating alloantibodies to recombinant FVIII [24]. Thus, the large insert capacity of this episomal EBV vector with a BAC backbone to transfer FVIII cDNA along with Wf cDNA simultaneously may be exploited in hematopoietic cells isolated from hemophiliacs for secretion of FVIII into the bloodstream. Due to their immediate accessibility from the peripheral blood, hematopoietic cells could be very attractive targets for gene therapy of not only hemophilia A, but also other clotting disorders, such as hemophilia B, von Willebrand disease, etc., where finally the responsible protein is present in the blood whatever be their site of expression. Recently, two similar studies using a baculostat lentiviral vector and a lentiviral vector containing B-domain-deleted FVIII cDNA under the control of a promoter consisting of the chicken β-actin promoter, CMV enhancer and a large synthetic intron (CAG), have also indicated the possibility of hematopoietic cells as targets for gene therapy of hemophilia A [5,35]. Another attractive target for gene therapy of hemophilia that is currently being intensely investigated is the bone marrow derived stromal cells [5]. In conclusion, our study provides proof of principal for the idea that EBV can be used to potentially target hematopoietic cells for human blood clotting disorders. It seems possible that further modification in the vector design and the choice of a helper-virus-free cell line could provide effective treatment with EBV-based episomal viral vectors.

Acknowledgements

This paper is dedicated to the memory of Dr. J-M. H. Vos who first initiated and inspired the second author (SB) to use EBV as a gene therapy vector. This is a tribute to him. We appreciate the kind help of Dr. A. Rickinson (UK) and Dr. G. Kline (Sweden) for providing the EBV-negative human B-cell lines. We want to specially thank Dr. J. A. van Mourik (CLB, The Netherlands) for the antibodies CAB CAG A and CAB CAG9 and also appreciate the extremely generous help of Dr. F Schefflinger (Baxter Hyland Immuno, Austria) for various literature and materials on hemophilia. Finally, we also thank the reviewers for their comments and suggestions.

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