Biotechnology and Applied Biochemistry

Stable and high-level production of recombinant Factor IX in human hepatic cell line

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Abstract.

Hemophilia B is a genetic disease of the coagulation system that affects one in 30,000 males worldwide. Recombinant human Factor IX (rhFIX) has been used for hemophilia B treatment, but the amount of active protein generated by these systems is inefficient, resulting in a high-cost production of rhFIX. In this study, we developed an alternative for rhFIX production. We used a retrovirus system to obtain two recombinant cell lines. We first tested rhFIX production in the human embryonic kidney 293 cells (293). Next, we tested a hepatic cell line (HepG2) because FIX is primarily expressed in the liver. Our results reveal that intracellular rhFIX expression was more efficient in HepG2/rhFIX (46%) than in 293/rhFIX (21%). The activated partial thromboplastin time test showed that HepG2/rhFIX expressed biologically active rhFIX 1.5 times higher than 293/rhFIX ($P = 0.016$). Recovery of rhFIX from the HepG2 by reversed-phase chromatography was straightforward. We found that rhFIX has a pharmacokinetic profile similar to that of FIX purified from human plasma when tested in hemophilic B model. HepG2/rhFIX cell line produced the highest levels of rhFIX, representing an efficient *in vitro* expression system. This work opens up the possibility of significantly reducing the costs of rhFIX production, with implications for expanding hemophilia B treatment in developing countries.

Volume 58, Number 4, July/August 2011, Pages 243–249 •
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Keywords: hemophilia B, recombinant human blood coagulation Factor IX, retroviral vector-mediated transfer, 293 and HepG2 cell lines

1. Introduction

Hemophilia B is an X-linked genetic disease of the coagulation system that affects one in 30,000 males worldwide. This disease is characterized by a deficient Factor IX (FIX) protein. Concentrates of FIX purified from human plasma (pdFIX) have been available since the 1960s to treat hemophilia B patients.

However, its production requires pooling of thousands of FIX units from human plasma to enable purification and production on a commercial scale. This requires large amounts of human plasma that are not always available. Also, the risk of virus contamination in blood samples should always be considered [1–3].

Recombinant human FIX (rhFIX) has been used as a safer alternative for hemophilia B treatment. The only commercial rhFIX (BeneFIX®, Wyeth Pharmaceuticals, New York, NY, USA) available is produced in Chinese hamster ovary cell lines co-transfected with the serine protease (PACE–FURIN) responsible for processing the Factor IX precursor polypeptide [3],[4]. The extensive posttranslational modifications that rhFIX undergoes, such as glycosylation and γ-carboxylation, require the use of cell lines equipped with such machinery. This represents the main limitation for obtaining an animal cell line able to produce high levels of active rhFIX. Experimental cell lines expressing rhFIX have also been developed by different groups [5–7].

The rate of rhFIX expression has been shown to vary significantly among cell lines [4],[6],[8–13]. By increasing selection pressure, Kaufman et al. [4] were able to produce cell lines with high mRNA/rhFIX rates but low amounts of active rhFIX protein. The addition of vitamin K to cell culture medium provided larger amounts of active recombinant protein [4],[12]. Even so, the amount of active protein generated by these systems is inefficient, resulting in high-cost production of rhFIX [14].

The developing countries do not have access to recombinant product. Brazil, for instance, spends around 120 million dollars/year on plasma-derived blood factors [15] (http://portal.saude.gov.br/portal/saude/hemobras/).

Abbreviations: aPTT, activated partial thromboplastin time; ELISA, enzyme-linked immunosorbent assay; FIX, Factor IX; HepG2, hepatic cell line; pdFIX, FIX purified from human plasma; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene fluoride; rhFIX, recombinant human Factor IX; RT-qPCR, real-time quantitative PCR; 293, human embryonic kidney 293 cells.

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Received 30 November 2010; accepted 15 April 2011
DOI: 10.1002/bab.32
Published online 9 August 2011 in Wiley Online Library (wileyonlinelibrary.com)

To test new cell lines capable of producing higher amounts of active rhFIX, we have investigated rhFIX expression and production in two different human cell lines. We tested human embryonic kidney 293 cells (293), which are commonly used to produce human recombinant proteins [16]. Then we worked with a hepatic cell line (HepG2) because FIX is primarily expressed in the liver. Additionally, this cell line bears a competent γ-carboxylation system, which is evidenced by its ability to synthesize vitamin K-dependent clotting factors such as factor X and protein C [17].

We obtained reasonable levels of rhFIX in the 293 cell line. On the other hand, our results reveal that higher levels of biologically active rhFIX can be obtained in HepG2 cells and that rhFIX secreted by these cells is functional *in vivo*.

2. Materials and methods

2.1. Construction of expression vector

Factor IX cDNA (access number NM_00133) was amplified by PCR from a human liver cDNA library (Clontech, Mountain View, CA, USA). The primers used were: P1FIXBamHI 5′-CGGGATCCTTAAGTGAGCTTTGTTTTTTTCCT-3′ and P2FIXHpaI 5′-AGCTTTGTTTAAACGTTAACACCATGCAGCGCGTGAACATGATC-3′. The 1.4-Kb PCR product was purified from agarose gel 1%, ligated into a retroviral vector pLXIN (Clontech), and its identity confirmed by sequencing and restriction digestion (*Bam*HI and *Hpa*I; Biolabs, Ipswich, MA, USA). The corresponding vector pLXIN–rhFIX grew in DH10β and the plasmid DNA was purified for further transfection.

2.2. Cells and cell culture condition

The 293 and HepG2 cell lines [American Tissue Culture Collection (ATCC), Manassas, VA, USA] were maintained at 37°C, 5% CO_2 in DMEM (Life Technologies, Carlsbad, CA, USA), and supplemented with 10% (v/v) FBS (Gibco, Carlsbad, CA, USA). The medium used for rhFIX expression was supplemented with 5 μg/mL of vitamin K (Kanakion® MM; Roche, San Francisco, CA, USA) for a period of 10 days. Twenty-four H before the biological activity and rhFIX antigen determination, the cells were treated or not with calcium ionophore (IO) 1 μg/mL (Sigma, St. Louis, MO, USA) and phorbol 12-myristate 13-acetate (PMA) 0.5 μg/mL (Sigma) and, before the assays, the supernatants were centrifuged at 10,000*g* for 60 Min at 20°C.

2.3. Virus-producing cell lines

EcoPack2^{TM}-293 cells (Clontech) were transfected with the pLXIN retroviral vector containing the rhFIX insert for packing. A total of 20 μg of pLXIN–rhFIX was used to transfect 2×10^7 cells by electroporation with the Gene-Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA), as described previously [18].

Retroviral particles were harvested from the EcoPack2^{TM}-293 cell medium 72 H after transfection. Harvested medium was filtered through 0.45-μm cellulose acetate filters (Millipore, Kankakee, IL, USA) to remove cell debris. The supernatant was collected and used to infect RetroPack2^{TM}-PT67 cells (Clontech) to increase retroviral titers. Stable rhFIX-producing cell lines were selected with 400 μg/mL G418 (Sigma) pressure. Vector-producing RetroPack2^{TM}-PT67 cells grew to 90% confluence in DMEM 10% FBS. In 48 H, the medium was collected, filtered with a 0.45-μm (Millipore) filter, and used to infect the human cell lines 293 and HepG2 when they reached 70% confluence.

2.4. Retroviral transduction

293 and HepG2 grew to 70% confluence in DMEM and were exposed to three cycles of infection with 1.10^5 viral particles/mL in the presence of 5.5 μg/mL polybrene (Sigma). After 5 days, 800 μg/mL G418 (Sigma) was added to the transduced cell lines. After 15 days, stable rhFIX-producing cell lines were obtained with a high stringency of G418 (2,000 μg/mL). Then, resistant cellular population of 293 and HepG2 were used in further experiments.

2.5. Quantification of vector DNA by real-time PCR

Genomic DNA was extracted from cells using a DNeasy Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The number of vector copies per genome was quantified by real-time quantitative PCR (RT-qPCR) from 100 ng of template DNA.

An rhFIX quantification standard curve was generated from 10-fold serial dilutions of pLXIN–rhFIX preparation. Target template serial dilutions and all experimental samples were duplicated. The specific primers for hFIX cDNA were: P5-5′-AGGAGACAGAACATACAGAGC-3′ and P3′-5′-CTTCCCCAGCCACTTACATA-3′. The following reaction conditions were 95°C for 10 Min; 95°C for 15 Sec, 60°C for 1 Min (40 cycles); 95°C for 15 Sec; 60°C for 15 Sec; 95°C for 15 Sec using a 7500 ABI Prism Sequence Detection System. Data were processed with the SDS 2.1 software package (PerkinElmer, Waltham, MA, USA; Applied Biosystems, Carlsbad, CA, USA).

2.6. RNA extraction and real-time quantitative PCR (RT-qPCR)

The RNA was prepared from the transduced cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The cDNA was obtained using SuperScript^{TM} II First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Specific primers for FIX cDNA (P5′-5′CAGTTGTCGCAGGTGA3′ and P3′-5′-TCATTAAGTGAGCTTTGTT-3′) were used. A total of 100 ng of RNA from each cell line was used in a SYBR^{TM} green RT-qPCR assay (7500 ABI Prism Sequence Detection System, Applied Biosystems), with 200 nmol/L of sense (5′-AGGAGACAGAACATACAGAGC-3′) and 200 nmol/L of antisense primers (5′-CTTCCCCAGCCACTTACATA-3′). The *GAPDH* gene was used as the internal control.

2.7. Intracellular rhFIX protein detection by flow cytometry

The transduced cells were fixed in paraformaldehyde 1%, permeabilized with 0.5% Tween (USB), and incubated in blocking solution (10% goat serum). The cells were incubated in PBS 1X containing 2% BSA and the primary polyclonal antibody Anti-Human Factor IX (Sigma). The cells were washed twice and incubated with secondary antibody FITC-conjugated swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) and

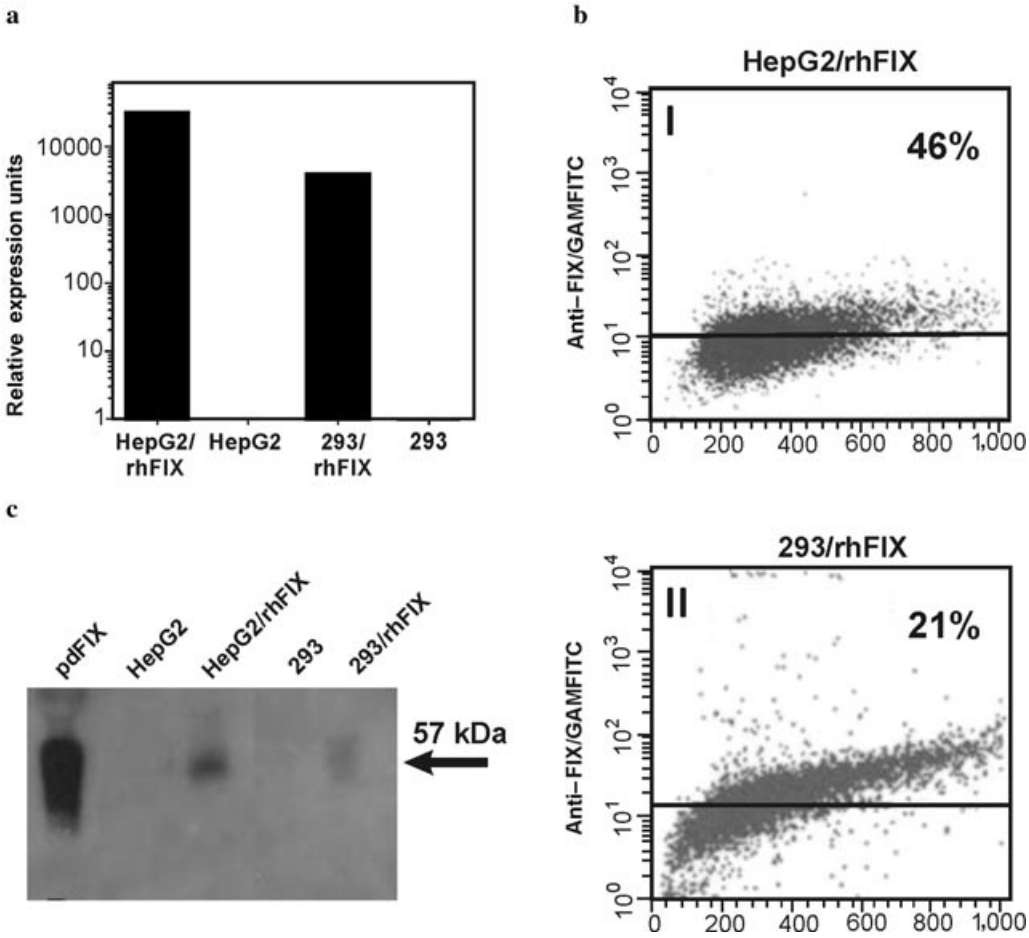

Fig. 1. rhFIX expression. (a) Quantification of rhFIX mRNAs from HepG2/rhFIX and 293/rhFIX cells by comparative analysis using qRT-PCR. (b) The percentage of cells expressing the intracellular rhFIX protein by flow cytometry. (I) Total of 46% HepG2/rhFIX positive cells, (II) total of 21% 293/rhFIX positive cells stained with anti-hFIX-FITC showing that after antibiotics selection a transgenic cell population was obtained. (c) Analysis of rhFIX protein in supernatant from HepG2 and 293 cells. Autoradiography of Western blot after immunodetection of the rhFIX protein (57 kDa) transferred to PVDF membrane. 1, positive control—pdFIX (1.5 μg/μL—Octanine®); 2, nontransduced HepG2 (15 μg/μL); 3, HepG2/rhFIX (15 μg/μL); 4, nontransduced 293 (15 μg/μL); 5, 293/rhFIX (15 μg/μL).

analyzed in the FACSort (Becton–Dickinson, Franklin Lakes, NJ, USA) using the CellQuest software.

2.8. Western blot of immunoreactive rhFIX

Western immunoblotting of rhFIX secreted by transduced cell lines was performed using a 5 × reduced culture medium. A total of 15 μg/μL of proteins and 1.5 μg/μL of pdFIX commercial (Octanine®; Octapharma, Charlotte, NC, USA) was separated by 12.5% SDS-PAGE stained with Coomassie B-Blue G250 or transferred to a PVDF membrane (polyvinylidene fluoride—Hybond™ P; Amersham Biosciences, Pittsburgh, PA, USA) using an electroblot system (PhastSystem, Pharmacia Biotech). The membrane was incubated with a rabbit anti-hFIX polyclonal antibody (Sigma) as primary antibody and chemiluminescence substrate (Western blot kit, Amersham Biosciences) as a detecting antibody. Commercial pdFIX (Octanine®; Octapharma) was used as standard.

2.9. Biological activity of rhFIX

The activity of rhFIX was measured by one-step clotting assay [activated partial thromboplastin time (aPTT)] employing human FIX deficient plasma using ACL 200 (Instrumentation Laboratory, Bedford, MA, USA), according to the manufacturer's instructions. We used the Verify plasma as reference (Organon Teknika, Burwood, VIC, Australia). An activity of 100% corresponds to 1 IU/mL of hFIX.

2.10. Determination of rhFIX antigen

Concentrations of rhFIX in supernatants were determined as rhFIX antigen by an enzyme-linked immunosorbent assay (ELISA), using the Asserachrom hFIX:Ag kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions.

2.11. Profiling of rhFIX by reversed-phase chromatography

To investigate complexity and relative abundance of rhFIX, reversed-phase chromatography profiling of conditioned media from rhFIX expressing cells and pdFIX (Octanine® — used as standard) was done on a Vydac C4 column (4.6 × 250 mm) (Vydac). The solvent system consisted of solvent A [0.1% TFA in water (v/v)] and solvent B [80% acetonitrile in water containing 0.09% TFA (v/v)]. Chromatography was performed at 1 mL/min at room temperature. The effluent was monitored at 220 nm. Fractions for further analysis by Western blotting were collected every minute during sample elution. The elution gradient is indicated in the respective figures (Fig. 1a).

2.12. Pharmacokinetic studies

Pharmacokinetic investigations were performed in FIX−/− mice (B6.129P2-*F9*^{tm1Dws}/J; Jackson Laboratories, Sacramento, CA, USA). All animals were maintained at temperatures between 21°C and 23°C and in a 12 H light–dark cycle. All experiments were approved by the local administrative authority and the animals received care in compliance with the Faculty of Medicine of Ribeirao Preto, University of São Paulo (CETEA/FMRP-USP). rhFIX-containing supernatants from HepG2/rhFIX cell culture were 5 × reduced and were administered intravenously at a dose of 2 IU/animal. Blood samples were drawn retro-orbitally at four different times after infusion (30 Min, 3, 6, and 24 H) and the hFIX plasma levels quantified. Plasma was prepared immediately after sampling and stored at −20°C until analysis. For determination of hFIX activity, the samples of two animals per group were analyzed for each time (30 Min, 3, 6, and 24 H) by hFIX aPTT test.

3. Statistical analysis

To evaluate differences between cell lines *in vitro*, we used the Student's *t*-test. Among *in vivo* groups, we calculated the area under the curve and used analysis of variance followed by a Bonferroni's multiple comparison test. We considered 0.05 as a significant value. All statistical analysis was done with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA).

4. Results

4.1. Retroviral vector encoding FIX leads to efficient stable transduction of human cell lines

The transduced cell lines with the retroviral vector pLXIN–rhFIX were selected by the marker gene neomycin. We found that rhFIX mRNA expression in HepG2/rhFIX was 7.6 times higher than in 293/rhFIX (Fig. 2a).

The intracellular rhFIX expression, measured by FACS analysis, showed that production of the recombinant protein was more efficient in HepG2/rhFIX (46%) than in 293/rhFIX (21%) (Fig. 2b). The percentage of intracellular rhFIX-expressing cells remained unchanged throughout cell culturing and expansion (total period of 4 months).

4.2. rhFIX is highly expressed in the cell lines after geneticin selection

Because the FIX is a vitamin K-dependent protein, the cells were treated with vitamin K for a period of 10 days prior to quantification of rhFIX biological activity. The biological activity of rhFIX secreted by human cells was assessed using the aPTT-based one-stage clotting assay. The rhFIX detected in HepG2/rhFIX was 1.8 times higher than 293/rhFIX without PMA and IO and 1.5 times higher than 293/rhFIX with PMA and IO treatment. The levels of rhFIX antigen, determined by ELISA, confirmed that secretion of rhFIX was higher in HepG2/rhFIX treated with PMA and IO. Table 1 shows the activity and antigen levels of rhFIX secreted in cell cultures. Nontransduced cells were used as the negative control, and human plasma was used as the positive control.

To determine whether different expression levels were due to the mean number of integrated viral vectors per cell, the number of integrated copies was assessed using quantitative Syber PCR of genomic DNA. The copy number of integrated viral vectors per cell ranged from 1.02 per HepG2 and 1.15 per 293 transduced cells. Note that retroviral vector copies in HepG2 and 293 transduced cells were remarkably similar.

4.3. Characterization of secreted rhFIX protein

Western blot analysis was performed to characterize the rhFIX released by cell lines transduced with the retroviral vector. The rhFIX was enriched from serum-free cell culture supernatants by ultrafiltration and analyzed by SDS-PAGE and immunoblotting. As a reference, we used pdFIX (Octanine®, Octapharma), which is composed of a 57-kDa chain [19],[20]. In both transduced cell lines, we detected the 57-kDa chain (Fig. 2c). No rhFIX protein could be detected in concentrated supernatants from cells that were not transduced.

To evaluate the complexity of the HepG2/rhFIX conditioned media and explore potential purification procedures for the rhFIX, reversed-phase chromatography profiling was performed. Supernatant of HepG2/rhFIX was dialyzed in an Amicon YM 3 system and injected into the chromatographic system (Fig. 1a). The elution profile of the HepG2/rhFIX supernatant indicates few major peaks.

The rhFIX was identified by Western blotting in fraction 3 (16 Min); this corresponds to fraction 1 (16 Min), which is the major peak of the Octanine® chromatogram (Fig. 1b).

4.4. Pharmacokinetics of rhFIX secreted by HepG2/rhFIX *in vivo*

The animals that received dpFIX and rhFIX showed a 50% decline in hFIX activity after 24 H. The comparison of the dpFIX and rhFIX activity profile shows a similar pharmacokinetic behavior (no statistical significance) (Fig. 3).

5. Discussion

In our study, we described the production of high levels of rhFIX protein using a retroviral expression system in two human cell lines. Our study also showed that this recombinant protein has

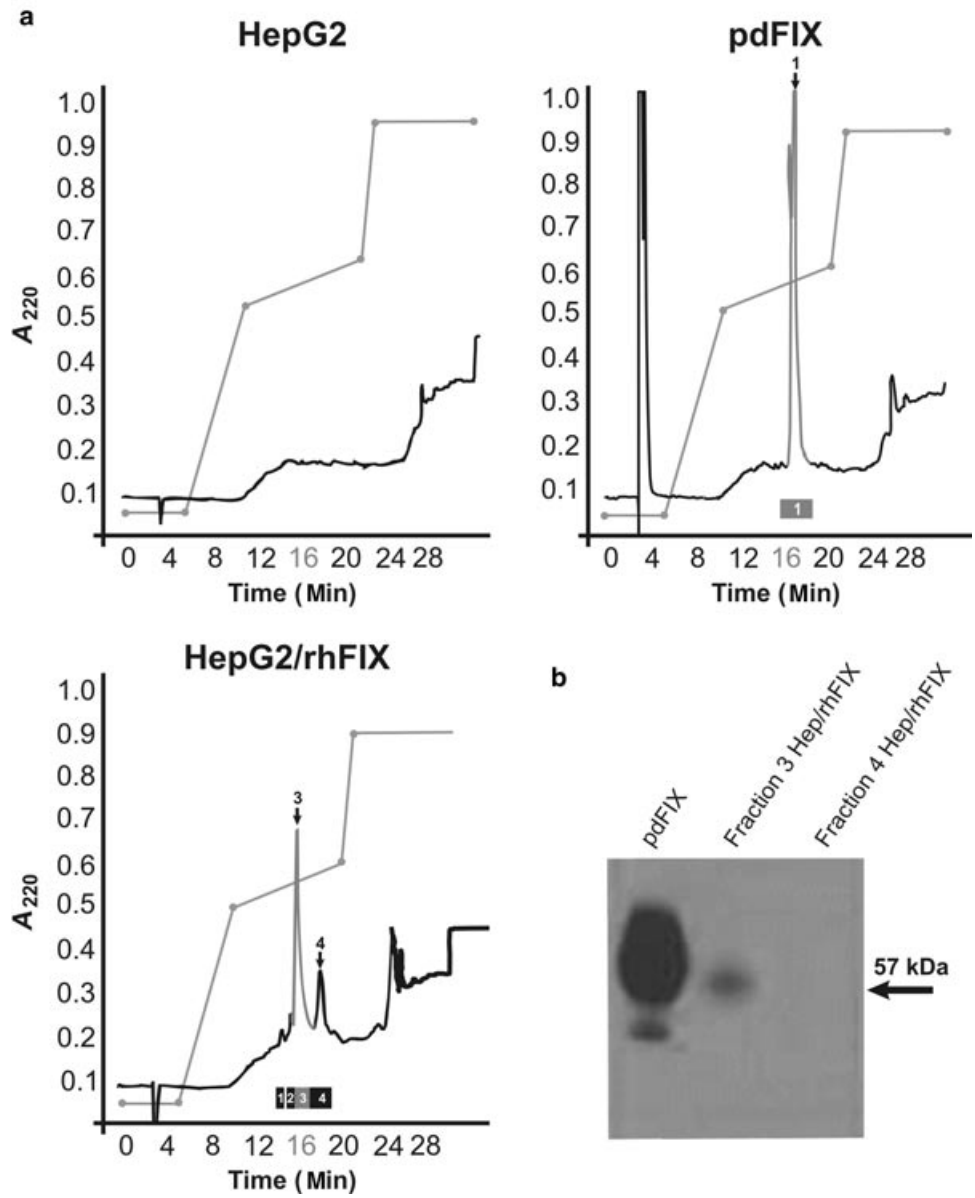

Fig. 2. Characterization of rhFIX expressed in the supernatant of HepG2/rhFIX. (a) A Vydac C4 HPLC column with a gradient of acetonitrile was used to characterize dpFIX, supernatant of HepG2, and HepG2/rhFIX. The fraction peaks are indicated by arrows. (b) Western blot using a polyclonal antibody Anti-hFIX. 1, pdFIX; 2, fraction 3 of HepG2/rhFIX; 3, fraction 4 of HepG2/rhFIX.

a pharmacokinetic profile similar to pdFIX when tested in a hemophilic B model.

We found that HepG2/rhFIX expresses the highest levels of rhFIX mRNA, intracellular protein. Nevertheless, the expression of the protein was stable throughout cell culture passages, in both cell cultures. Previous experience by our group (unpublished data) has shown that the use of high stringency selection with geneticin produces stable production of recombinant protein.

Analysis of the supernatant of both transduced cells showed an immunoreactive band of 57 kDa corresponding to hFIX, which suggests that the protein found in the supernatant is intact. Recovery of rhFIX from the HepG2/rhFIX by reversed-phase chromatography was straightforward.

The analysis of rhFIX coagulation activity showed that the transduced human cell lines were able to produce biologically active rhFIX. However, HepG2/rhFIX produced higher levels of the biologically active protein when compared with 293/rhFIX.

The low expression observed in 293/rhFIX might be due to the low percentage of carboxylated rhFIX, once these cells present an inefficient γ-carboxylation mechanism [21]. Recombinant factor X (rFX) expression in 293 cell line can be improved by enhancing the carboxylation process in this cell line [21]. Previous studies have shown that 293 secretes a mixture of

Table 1
hFIX activity and antigen in transduced cells and human plasma

	FIX–Activity mIU/10^6 cells/24 H		FIX–Antigen mIU/10^6 cells /24 H	
Cell line	Without PMA and calcium ionophore 1	With PMA and calcium ionophore 1	Without PMA and calcium ionophore	With PMA and calcium ionophore 1
HepG2 non-transduced	0 ± 0	4 ± 1[b]	0 ± 0	0 ± 0
HepG2/rhFIX	590 ± 40[a,e]	1080 ± 28[b,d,e]	40.9 ± 2.6	245 ± 2.1
293 nontransduced	0 ± 0	5 ± 2[c]	0 ± 0	0 ± 0
293/rhFIX	320 ± 110[a,f]	730 ± 89[c,d,f]	16.1 ± 2.3	164 ± 1.6
Human plasma	1.06 ± 0.1 IU/mL		0.68 ± 2.9 IU/mL	

Cell lines with *P* values represented by the same letter have significant differences; [a]$P = 0.0144$; [b]$P = 0.0004$; [c]$P = 0.0019$; [d]$P = 0.0158$; [e]$P = 0.0059$; and [f]$P = 0.0212$.

Presented data are the mean of cultures in duplicate.

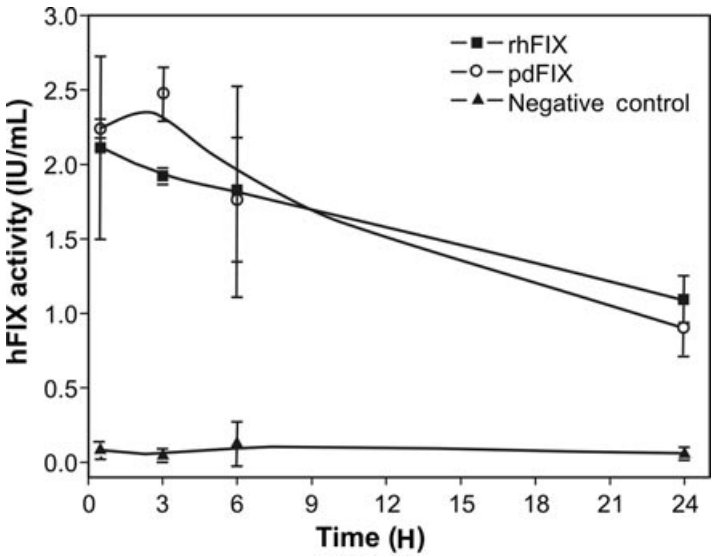

Fig. 3. rhFIX biologically active in the plasma of hemophilia B mice after intravenous infusion. Pharmacokinetic profile of rhFIX and pdFIX (Octanine®) after intravenous infusion via tail vein (2 IU/animal). Average values for 2 (pdFIX), 2 (rhFIX), and 2 (negative control) animals per group are shown. A statistically significant difference ($P < 0.05$) among three animal groups using ANOVA is also shown.

partially and completely γ-carboxylated proteins [22],[23]. To increase production of the protein of interest, cell cultures have been treated with inducing agents such as PMA [24].

Blostein et al. [25] reported the production of 100 mIU of FIX from 10^6 cells in 24 H in 293 cell culture. Dadehbeigi et al. [26] also showed the expression of biologically active rhFIX in 293 cells (62 mIU/10^6 cell/48 H). Using our expression system, combined with treatment with PMA and IO, we were able to produce levels of rhFIX secreted by 293 cells that were 7–11 times higher than those produced by others [25],[26].

In treated HepG2/rhFIX cell cultures, we were able to produce eight times more rhFIX antigen than was produced by Chen et al. [13] using different retroviral vectors and the same cell line, and 1.3 times more rhFIX than produced by Gordon et al. [9], which also used HepG2 cells and retrovirus vector. This higher expression may be associated with inducers of protein secretion treatment, which were used in this work. In addition, the biological activity of treated HepG2/rhFIX was two times higher than that achieved by Wajih et al. [12] in BHK cells using the same retroviral vector. This increase may be attributed to the fact that the HepG2 cell line has the carboxylation system required for generating active FIX [17]. In summary, when compared with other expression systems, our HepG2/rhFIX cell line produced the highest levels of rhFIX, representing an efficient *in vitro* expression system.

The rhFIX produced by this cell line has a coagulant effect and kinetic profile similar to that of pdFIX (Octanine®) when infused intravenously in hemophilic B mice. Thus, the ability to correct clotting in hemophilic mice suggests that HepG2-synthesized rhFIX is biologically active also *in vivo*. Furthermore, the decline of rhFIX pharmacokinetics was also similar to that obtained in studies done in dogs and in hemophilic B mice after intravenous infusion of rhFIX and pdFIX [27],[28].

Here we describe the development of a biotechnological platform for the production of recombinant protein using human cell lines. The next challenge is to obtain a large production of this protein in HepG2/rhFIX in serum-free suspension cultures. This work opens up the possibility of significantly reducing the costs of rhFIX production, with implications for expanding hemophilia B treatment in developing countries.

Acknowledgements

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant number 2008/04264-4), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant number 680145/2001-0), and Financiadora de Estudos e Projetos (FINEP, grant number 01.05.0691-02), Brazil.

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