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Expression of functional recombinant human factor IX in milk of mice

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Abstract Human factor IX is synthesized in the liver and secreted in the blood, where it participates in a group of reactions involving coagulation factors and proteins that permit sanguinary coagulation. In this work two lines of transgenic mice were developed to express the *FIX* gene in the mammalian glands under control of milk β-casein promoter. The founding females secreted the FIX in their milk (3% total soluble protein). The stable integration of transgene was confirmed by southern blot analysis. The presence of the FIX recombinant protein in the milk of transgenic females was confirmed by western blot and the clotting activity was revealed in blood-clotting assays. The coagulation activity in human blood treated with recombinant FIX increased while the time of coagulation decreased. Our results confirm the production of a large amount of recombinant biologically active FIX in the mammary gland of transgenic mice.

Keywords Expression · Recombinant human factor IX · Transgenic mice

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Introduction

Hemophilia B or Christmas disease is a recessive X-linked disease characterized by abnormal production of human coagulation factor IX. In Brazil there is an estimated incidence of 1 in 8,000 born, and 15% of these have hemophilia B. Human factor IX is a 55 kDa single chain vitamin K-dependent plasma zymogen which has a serine protease function and plays a key role in the intrinsic coagulation pathway. This pathway is a series of enzyme-mediated cleavages by means of plasma factors of peptide chain fragmentation. Upon activation of FIX to FIXa by FXIa, an 11 kDa activation peptide is removed from factor IX molecule by cleavage of two peptide bonds. These changes allow the activation of factor IX and are crucial to prothrombin and fibrinogen production and blood clotting (Di Scipio et al. 1978).

Factor IX is synthesized in the liver's parenchymal cells and requires a posttranslational vitamin K-dependent modification in order to work

appropriately. Benefix, a recombinant FIX purified from transgenic chinese hamsters ovary cells, was the first recombinant drug successfully destined to hemophilia B treatment and demonstrated to be stable and safe both in vitro and under clinical conditions (Chowdary et al. 2001).

Besides this important effort, Christmas disease treatment by injection of either recombinant or purified FIX from human healthy donors' plasma (the most usual treatment form), still incurs on heavy costs and prion and virus contamination risks. Besides, human-derivate factors are unstable and require frequent injections. Today, Brazil is totally dependent on the importation of human factor IX molecule from pooled plasma.

Transgenic animals are indicated as potential successful bioreactors for complex heterologous protein biosynthesis, such as FIX, since high yields of protein and structural quality are generally associated with those systems (Houdebine 1994; Wall 1996). Another advantage of alternative based production of recombinant drugs in transgenic mammals is the ability to provide post-translational modifications correctly, which allows folding assembles and glycosilation similar to those present in humans (Larrick and Thomas 2001). The mammary gland can currently be considered one of the most efficient bioreactors (Colman 1996; Rudolph 1999). Extensive studies have demonstrated the possibility of the production of a great variety of recombinant proteins in milk, many of them complex proteins, such as: human IGFI (Zinoveiva et al. 1998), hGH (Devinoy et al. 1994), human lysozime (Lee et al. 1998), human lactoferrine (Platenburg et al. 1994), human erythropoietin (Sohn et al. 1999), human parathyroid hormone (Rokkones et al. 1996), recombinant factor VIII in milk of transgenic rabbit (Chrenek et al. 2007). These examples demonstrate the capacity of the mammary gland to synthesize, to mature and to secrete active biologically recombinant proteins.

Transgenic mice lines are attractive because of a series of advantages like the shortest reproduction cycle even when compared to other mammals, fast female lactation, high milk protein content and the relatively lower costs of maintaining the animals. Moreover, this model system will make future applicability possible in large animals, such as cattle and goats, used as efficient bioreactors. In the present work we report the expression of human coagulation factor IX in transgenic mice milk and the evaluation of independent line level expression and biological function of the recombinant molecule. For human FIX expression in transgenic mice, the protein coding sequence was flanked by the β-casein promoter, a strong mammary gland tissue-specific regulatory sequence and microinjected in mice pronuclei.

Material and methods

Human factor IX expression vector

The code region of the FIX (coding for the human factor IX) gene (GeneBank accession number: XM010270) (Yoshitake et al. 1985) was amplified by PCR from a cDNA library (λ TriplEx, Clontech, USA) from human liver. The fragments were cloned into the pGEM-T Easy (Promega, USA) vector for PCR products. Primer pair BCproFIX [5′-G**CTCGAG**AT-**GGCAAAGGTCCTCATCCTTGCCTGCCTGG-TGGCTCTGGCCCCTTGCA**ACAGTTTTTCTTGA TCATGAAA-3′; including the sites for XhoI [underlined] and the sequence of the secretion signal of the bovine beta-casein (in bold)] and FIXstop3C [5′-CCTT<u>CTCGAG</u>CCATCTTTCATTAAGTGAGC-3′; including the sites for XhoI (underlined)] was used to amplify a 1,378-bp fragment from the FIX gene. PCR reactions were carried out in a thermocycler in 50 µl containing 10 ng DNA, 60 mM Tris/H_2SO_4 (pH 8.9), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 250 nM each dNTP, 200 nM each primer, and 5 U of platinum Taq DNA polymerase high fidelity (Invitrogen, USA). The mixture was treated at 95°C (5 min) and subjected to 35 cycles of amplification (95°C/1 min, 55°C/1 min, and 68°C/1 min), with a final elongation cycle of 5 min at 68°C. PCR products were sequenced by using universal M13 and T7 primers on automatic sequencer (ABI Prism1 3700). The 1,378-bp fragment from the FIX gene was cloned into the XhoI restriction site of the pBC1 mammalian expression vector (Invitrogen, USA) to generate the vector pBC1FIX, which was used for pronuclear microinjection.

DNA preparation for generation of transgenic mice

The plasmid pBC1FIX was digested with SalI and NotI to eliminate the prokaryotic ampicilin sequence.

The fragment containing the *FIX* sequence was separated by 0.8% agarose gel electrophoresis and purified from gel with Qiaex II gel extraction kit (Qiagen, USA). The elution of DNA from the column was carried out with microinjection buffer (MIB-8 mM Tris/HCl, pH 7.4, containing 0.15 mM EDTA). The concentration was adjusted to 3 ng/μl in MIB, and used for production of transgenic mice. All animal manipulation and surgical procedures were carried out at Embrapa Recursos Genéticos e Biotecnologia, Hospital de Apoio de Brasília and Universidade Federal de São Paulo in accordance with the ethical standards of the institutional committees and the Helsinki Declaration.

Superovulation

B6CBAF1 female mice (4–5 weeks old) were superovulated by intraperitoneal (i.p.) injection of 5 IU pregnant mare's serum gonadotrophin (PMSG—Sigma, USA). Approximately 48 h later, 5 IU human chorionic gonadotropin (hCG—Sigma, USA) were administered, also by i.p. injection, and coupled overnight with B6CBAF1 male mice.

Collection of zygotes

The pronuclear-stage embryos were collected 20 h after the hCG injection. The cumulus cells were removed by placing the zygotes in M2 medium (Sigma, USA) complemented with hyaluronidase (0.1% w/v) (Sigma, USA) for 2 min. The embryos were washed several times in pure M2 medium to remove hyaluronidase and were maintained in M16 culture medium (Sigma, USA) at 37°C under 5% CO_2 until microinjection.

Pronuclear microinjection

Approximately 3 picolitre of exogenous DNA solution was injected into one of the pronuclei with the aid of a pair of manipulators at a 400× magnification, in an inverted microscope with Normarski differential interference contrast optics. The injection took place in a small drop of M2 medium covered by mineral oil (Sigma, USA) as previously reported (Chrenek et al. 2007). After that, the surviving embryos returned to M16 medium in the CO_2 incubator.

Embryo transfer

Microinjected embryos (15 to 30 zygotes per foster mother) were transferred into the oviduct of pseudo-pregnant (Swiss) females on the day of microinjection. The pseudopregnancy of females was induced by mating with vasectomized (Swiss) males (Chrenek et al. 2007).

PCR analysis

Genomic DNA was purified using the commercial Wizard Genomic DNA Purification kit (Promega, USA) and used as a template in the reactions containing specific primers that pared with the internal sequence of the *human factor IX* gene. Primer sequences FIXAL256: 5′-GATGGAGATCAGTGTGAGTC-3′ and FIXAL 751c: 5′-TAACGATAGAGCCTCCACAG-3′ were utilized to amplify a 495 bp sequence. DNA was subjected to 35 cycles of amplification (94°C/2 min, 94°C/1 min, 58°C/1 min, 72°C/1 min and 72°C/7 min) carried out in 25 μl aliquots containing 10 mM Tris/HCl (pH 8.4); 50 mM KCl; 2 mM $MgCl_2$; 160 μM each dNTP; 2 μM each primer; 2 U *Taq* polymerase (Invitrogen, USA) and 20 ng genomic DNA.

Recovery and total protein extraction

Milk samples were taken from lactating transgenic and non-transgenic female mice on day 7 of first lactation. In order to stimulate let-down of milk, an intramuscular injection of 5 IU oxytocin (Sigma, USA) was applied 30 min before milk collection. The samples were either subjected to further analysis or stored at −80°C until used.

Recombinant protein extraction from the milk of the transgenic and non-transgenic animals was carried out by homogenization 1:1 (v/v) in extraction buffer (NaH_2PO_4 50 mM, NaCl 20 mM, PMSF 2 mM and DTT 10 mM, pH 7.5) for 1 h at 4°C, followed by centrifugation (5,000g for 20 min at 4°C) and the upper lipid layer and the supernatant removed. Total protein quantification was carried out by the Bradford method.

Southern blot analysis

Genomic DNA (15 μg) isolated using Wizard Genomic DNA Purification kit (Promega, USA) was

digested with *Kpn*I (100 units), separated on 1% agarose gel and transferred to a nylon membrane (Hybond N$^+$). The hybridization was carried out using the PCR-generated 510 bp *FIX* probe, labeled with $\alpha[^{32}P]$-dCTP (110 TBq/mol) using a random primer DNA labeling kit (Pharmacia Biotech, USA) according to the manufacturer's instructions.

Western blot analysis

Total protein (60 μg) was extracted from transgenic mice milk and 350 ng human factor IX (Sigma, USA) were resolved in a 12% (v/v) SDS-PAGE mini gel, electrotransferred to Hybond N$^+$ nitrocellulose membrane (Hybond) under 100 mA for 50 min, followed by 4°C overnight blocking in Tristy buffered saline solution (20 mM Tris base, 137 mM NaCl, pH 7.6) containing 5% (v/v) dry milk and 0.1% Tween 20.

The membrane was incubated with rabbit igG policlonal anti-human factor IX diluted 1:2,500 in blocking buffer, for 4 h at 25°C. Secondary antibody incubation was performed for 3 h at 25°C with goat anti-rabbit igG conjugated with alkaline phosphatase (Bio-Rad) in a dilution of 1: 5,000 in blocking buffer. Alkaline phosphatase activity was monitored by 10 ml detection buffer consisting of 100 mM Tris/HCl pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$, 66 μl NBT 50 mg/ml (Nitro blue tetrazolium) and 33 μl BCIP 25 mg/ml (5-bromo-4cloro-3-indolil phosphate). Reaction was stopped by Tris/EDTA 20 mM and the colored product was detected.

Bioactivity assay

The functional activity of FIX in mice milk samples was determined by assaying its ability to restore clotting activity using FIX-depleted human plasma together with the respective lacking factor. The partial thromboplastin time was interpreted using a reference curve obtained with dilutions of standard human plasma or a normal plasma pool mixed with the deficient plasma.

The samples (transgenic mice milk and non-transgenic mice milk) were diluted to 160 pg/μl of factor IX. 100 μl each diluted milk sample was added in 100 μl of factor IX-deficient plasma plus 100 μl activated cephalin. After 5 min of incubation at 37°C, 100 μl 0.025 M CaCl$_2$ was added, and the coagulation time determined.

Results

Transgenic mice

F$_2$ Hybrid zygotes produced by mating male and female F$_1$-hybrids (B6CBAF1) were microinjected with pBC1FIX vector and yielded thirty-four litters. PCR and southern blot analysis revealed two transgenic founders. The transgenic male founder was responsible for the continuity of line 1, and the transgenic female founder for line 2. In order to generate the progeny of these transgenic animals, the male and the female founders were mated with wild type animals of the C57bl/6 strain. As a result, 13 animals were generated on the F$_1$ generation, 9 animals from line 1, and 4 from line 2. F$_1$ progeny was submitted to PCR analysis, and the positive females were mated with male wild type mice for the attainment of the F$_2$ generation. Seven days after birth of the F$_2$ generation, the milk of the founders and F$_1$ female mothers was collected in order to analyze the FIX recombinant protein expressed in their milk.

PCR analysis (Fig. 1) revealed the presence of a 495-bp fragment from the factor *IX* transgene coding
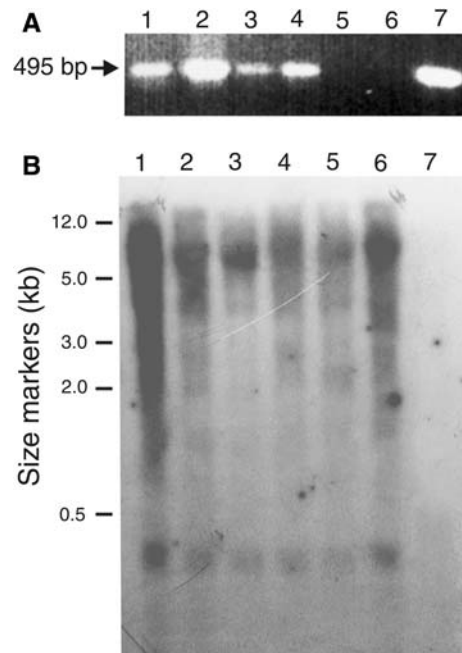

Fig. 1 (a) PCR screening of transgenic mice for the presence of the internal sequence of the *human factor IX* gene. Lanes 1, 2, 3 and 4: transgenic mice; Lane 5: non-transgenic mice; Lane 6: internal negative control; Lane 7 Plasmid vector. (b) Southern blot analysis of transgenic animal lines. Lanes 1 to 6: transgenic mice; Lane 7: non-transgenic animal
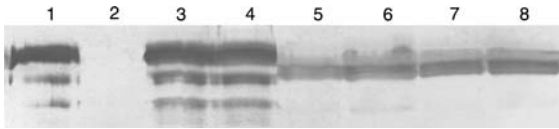
Fig. 2 Western blot for detection of FIX in the milk samples from transgenic mice females expressing the *human factor IX* gene. Lane 1: 350 ng of purified FIX protein (Sigma, USA); Lane 2: 100 μg total milk protein of non-transgenic female; Lanes 3 and 4:100 μg of transgenic founders lines 1 and 2; Lanes 5 and 6: 100 μg of F_1 progeny from line 1; Lanes 7 and 8: 100 μg of F_1 progeny from line 2

in both mice founders and progenies. Southern blot analysis confirmed the integration of the *FIX* transgene (Fig. 1). DNA isolated from a non-transgenic line did not hybridize with the *FIX* cassette probe.

Western blot analysis

In milk samples obtained at day 7 from two lactating females from distinct lines, a band corresponding to 50 kDa was detected by western blot analysis. This signal was not detected in non-transgenic milk (Fig. 2).

Functional activity of FIX in mice milk

Bioactivity assay was performed in order to detect the activity of FIX recombinant protein in percentage rate of its clotting action. It was found that a high level of concentrated active protein was encountered per microliter in mice milk. Milk of transgenic founder line 1 (F_0), milk of transgenic founder line 2 (F_0), normal human plasma, hemophilic plasma supplemented with 100 pg/μl purified FIX from human plasma (Sigma, USA) and milk of non-transgenic female were evaluated. The coagulation activity detected by percentage was 1.7, 3.1, 100, 0.7 and 0, and the time of coagulation was confirmed at 76, 69, 37.6, 87 and 141 s, respectively (Table 1).

Discussion

Transgenic animals are capable of producing biologically active recombinant proteins at high levels. With the use of the expression vector pBC1, specific for the genetic transformation of mammal cells, the recombinant proteins are secreted at high levels in the

Table 1 *FIX* recombinant coagulation activities in the milk of transgenic and non-transgenic mice

Samples	Method		
	Western blot	Coagulation activity (%)	Thromboplastin time (s)
Milk of transgenic founder line 1 (F_0)	+	1.7	76
Milk of transgenic founder line 2 (F_0)	+	3.1	69
Normal human plasma	+	100	37.6
Hemophilic plasma supplemented with 100 pg FIX/μl (Sigma, USA)	+	0.7	87
Milk of non-transgenic female	−	0	141

transgenic animal's milk. Based on this, our constructed expression vector is an integrative vector that confers stable integration on the host genome and expression of recombinant protein in the milk of transgenic animals detected by southern blot, western blot and bioactivity analysis.

We used the microinjection method to produce transgenic mice. It is the most efficient technology used in generation of transgenic mice for gene addition (Watanabe et al. 2007) compared with other systems (Huszar et al. 1985; Jahner et al. 1985; Lavitrano et al. 1989). Our transgene integration efficiency was 6% considering the presence of two founders in 34 litters born. However, this technique has proven to be far less efficient in farm animals (Wall 2001). Since this technique results in randomly inserted transgenes, transgenic mice were screened by PCR and confirmed by southern blot analysis in order to evaluate the presence of the transgene introduced in the founders as well as in the progeny. We have studied transgenic integration in bovine cells (Lisauskas et al. 2007) to carry out gene-target integration to produce desired recombinant protein expression levels instead of randomly integrated and variable expression.

Our results showed that the amount of recombinant polypeptide secreted by mammary gland cells of

founder 2 reached up to 2% of total soluble milk protein content (data not shown), which indicates a satisfactory expression level modulated by the beta-casein promoter. Korhonen et al. (1997) generated several transgenic mouse lines and rabbits expressing human erythropoietin efficiently in their milk (up to 0.3 mg/ml in mice and up to 0.5 mg/ml in rabbits) with bovine beta-lactoglobulin as promoter.

Western blot results suggest the maintenance of the correct molecular mass of secreted FIX presented in both founders and F_1 females' milk; they also demonstrate the efficiency of the secretion strategy provided by the beta-casein secretion signal. Three band patterns presented by SIGMA's plasma purified FIX and recombinant mice FIX probably reflect the activation of FIX into FIXa by the cleavage of two peptide bonds of the 55 kDa single chain (upper band), releasing the FIX 44 kDa heavier chain (intermediate band) and the 11 kDa light chain (lower band). The high concentration in mice milk of vitamin K, which is important in effecting coagulation, corroborates the hypothesis of correct activation of FIX zymogen, correlated to western blot band pattern and further proof by coagulation bioassay.

In our study, to prove the efficiency of the FIX recombinant protein activity further, bioassays were performed with purified recombinant protein from transgenic mice milk. Activity of the recombinant coagulation factor IX protein was observed in its coagulant action, demonstrating the protein's function produced in heterologous system. Milk of transgenic founder line 1, transgenic founder line 2, normal human plasma, hemophilic plasma and milk of non-transgenic female were compared. The activity of the protein was detected in percentage rate of its clotting action. It was found that a high level of concentrated active protein was encountered per µl in mice milk.

Transgenic technology is especially attractive for the recombinant protein production necessary on a large scale, due to units of required dosages, multiple administration, application in the population on a large scale, or need for industrial production. The annual market of large-scale recombinant proteins, plasma factors VIII and IX, is approximately 5–10 kg. In accordance with the World Federation of Hemophilia (Montreal, Canada), the cost incurred in supplying the current need for factor IX is approximately US$190 million in the United States. For factor VIII, this cost is approximately US$ 2 billion. The potential market for these products has been limited for the plasma supplement.

The final stage of the process that consists of the purification of recombinant proteins from milk generally does not present particular difficulties. Among the possible biological contaminants, the prions are the main constrain. However, evidence from the World Health Organization emphasizes that prions have not been detected in association with milk and semen, when closed herds are prioritized (Rudolph 1995; Gavin 2001). This work will form the foundation for the production of transgenic goats and cattle expressing recombinant human factor IX in the milk.

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