Gene Therapy for Hemophilia*

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Key Words

Hemophilia A · Hemophilia B · Gene therapy protocols ·
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Summary

Hemophilia A and B are inherited coagulopathies caused by mutations in the factor VIII (FVIII) and factor IX (FIX) gene, respectively. Although today, both diseases are well or excellently be treatable by intravenous substitution of FVIII and FIX concentrates, considerable efforts are being made to develop gene therapy protocols for hemophilia aimed to improving patients' quality of life and to reducing the costs of substitution therapy. Gene therapy aims to correct gene defects and is based principally on the introduction of the intact FVIII or FIX gene into somatic cells. This procedure requires viral transfer vectors. So far, sufficient and even high gene therapeutically expressed concentrations of FVIII/FIX could only be achieved in animal studies, while clinical protocols for humans had to be preliminarily stopped due to inefficacy and side effects. Nevertheless, based on the success of the animal studies, it can be expected that similarly high expression rates can also be achieved in humans. With view to the fact that in the present situation patients are at high risk of developing thrombotic events, this study presents a doxycycline inducible expression system for human FIX integrated within an adenoviral gene transfer vector. After intravenous injection of modified adenoviral vectors in mice, the efficacy of the regulation was demonstrated by measuring the FIX antigen concentration as well as FIX activity in mouse blood. Additionally, by measuring the D-dimer concentration in mouse blood and correlating these values with the gene therapeutically expressed human FIX concentration, we could demonstrate, for the first time, that high expression levels of human FIX in a gene therapy protocol increased the blood procoagulant activity in animals.

Schlüsselwörter

Hämophilie A · Hämophilie B · Gentherapieprotokolle ·
Faktor IX-Expression

Zusammenfassung

Hämophilie A bzw. B sind vererbare Blutgerinnungskrankheiten, die durch Mutationen im Faktor-VIII(FVIII)- bzw. Faktor-IX(FIX)-Gen verursacht werden. Obwohl heutzutage beide Krankheiten hervorragend durch intravenöse Substitution von FVIII- und FIX-Konzentraten behandelt werden können, werden beträchtliche Anstrengungen unternommen, eine Gentherapie für die Hämophilie zu entwickeln. Ziel ist es, die Lebensqualität der Patienten weiter zu verbessern und die Kosten der Therapie zu reduzieren. Das Prinzip der Gentherapie beruht dabei darauf, dass intakte Kopien des FVIII- bzw. des FIX-Gens in somatische Zellen eingebracht werden, um den Gendefekt zu kompensieren. Für eine effizientes Einbringen der Gene werden allerdings virale Vektorsysteme benötigt. Auch wenn bisher mittels der viralen Gentransfersysteme extrem hohe FVIII/FIX-Konzentrationen nur in Tierversuchen erreicht werden konnten und die am Menschen durchgeführten klinischen Studien aufgrund von Ineffizienz und einigen Nebenwirkungen vorübergehend unterbrochen wurden, kann davon ausgegangen werden, dass ähnlich hohe Expressionen auch bald im Menschen erreicht werden können. Da zu hohe FIX-Konzentrationen aber für Menschen ein hohes Thromboserisiko bedeuten, stellt diese Studie ein in einem adenoviralem Gentransfervektor integriertes Doxycyclin-abhängiges Regulationssystem für die Synthese von humanem FIX vor. Die Funktionsfähigkeit dieser Steuerung konnte in vivo nach intravenöser Substitution des modifizierten adenoviralen Gentransfervektors in Mäuse durch Messung der FIX-Antigenkonzentration und der FIX-Aktivität im Mausblut demonstriert werden. Durch Messung der D-Dimere im Mausblut sowie der Korrelation dieser Werte mit den gentherapeutisch exprimierten FIX-Konzentrationen konnten wir zusätzlich zum ersten Mal nachweisen, dass hohe, in einem Gentherapieprotokoll für Tiere erreichte FIX-Werte zu einer erhöhten Blutgerinnungsaktivität führen können.

*Dedicated to Prof. Dr. Peter Hanfland, Bonn, on the occasion of his 65th birthday.

Introduction

Blood coagulation is part of an important host defense mechanism termed hemostasis. Upon vessel injury, platelets adhere in the subendothelial tissue and then aggregate to form the primary hemostatic plug. The platelets stimulate local activation of plasma coagulation factors, leading to generation of a fibrin clot that reinforces the platelet aggregate. Activation of coagulation factors occurs principally via two pathways. The extrinsic pathway is the principal initiator of coagulation and is instigated upon vascular injury leading to exposure of tissue factor. The intrinsic pathway is necessary to sustain the coagulation response. It is initiated when contact is made between blood and exposed endothelial surfaces and leads to formation of the enzymes XIIa and kallikrein. Both pathways leads to the activation of factor X and thereafter converge on a common sequence of final steps to form the fibrin clot. A deficiency or dysfunction of either factor VIII (FVIII, hemophilia A) or factor IX (FIX, hemophilia B), which are members of the intrinsic pathway, compromises the coagulation response and leads to bleeding following vascular injury. In healthy individuals, both proteins are synthesized in liver cells which secrete the intact molecules within the blood vessels.

Dysfunctional FVIII or FIX proteins result from mutations in the respective gene [1, 2]. Fortunately, patients suffering from one of these blood coagulation disorders can be treated by either FVIII or FIX concentrate. Although this substitution therapy enables patients to live an almost normal life, hemophilia A and B are for several reasons ideal diseases for the development of (different) gene therapy protocols: i) FVIII and FIX are well characterized and can be monitored easily in blood gained from patients. ii) Due to substitution therapy, hemophiliacs have a long life expectancy, which helps to determine side effects of gene therapy protocols. iii) Gene therapy protocols can be tested in different hemophilic animals (mice, canines, rabbits). iv) Gene therapy would not only spare the patient the burden of regular replacement therapy but possibly also reduce treatment costs.

As it is not yet possible to repair the molecular defect of the FIX and FVIII genes within liver cells, present gene therapy attempts to bring intact FVIII or FIX genes into the cells. This technique, called gene substitution, enables cells to produce again the missing blood coagulation factor. However, since cells are not able to pick up intact genes, FVIII or FIX genes have to be packed into transfer vehicles (vectors) able to enter cells efficiently. Nowadays, the most efficient vectors are viral vectors (e.g. adeno-associated virus, retrovirus, adenovirus). But what makes viruses so suitable to transfer genes into cells?

Viruses are in general composed of nucleic acids (DNA or RNA) enclosed by viral capsid proteins. Viruses are obligate parasites, and thus they can propagate only in a living host cell. Therefore, viruses have developed an efficient infection process that allows them to enter cells and deliver their

Table 1. Expression results of FIX activity (FIX:C) and FVIII activity (FVIII:C) in animals treated with different kinds of transfer vectors containing the FIX and the FVIII gene, respectively

Animal	Gene transfer system	FIX:C, %	Expression time, months
Mouse	adenovirus [3]	5,000	3
Canine	adeno-associated virus [4]	10	12
FVIII:C, %			
Mouse	adenovirus [5]	150	9
Canine	adeno-associated virus [6]	116	>12

genome (DNA or RNA) to the host cell nucleus. Inside the nucleus, a few viral genes are transcribed, and information of synthesized mRNA is translated into proteins within the cytoplasm of the cells. These viral proteins divert and accelerate the cellular DNA replication and protein machinery for the replication of the viral genome and the synthesis of the capsid proteins, respectively. The viral genome and the capsid proteins then assemble in the cytoplasm to generate a complete infectious virus that can exit the cell and infect new cells.

Taking advantage of the ability of viruses to enter host cells and deliver their load of nucleic acids to the nucleus, the viral genome has been manipulated to delete some key genes that rendering the virus replication deficient and making room for foreign genes (FVIII or FIX). Although the manipulated viral genome cannot replicate in the host cells, it still can be replicated in vitro in special production cell lines that are able to provide the deleted genes in trans.

So far, many viruses (e.g. adenovirus, adeno-associated virus) have been manipulated for use as a vehicle or vector for gene transfer, and the appropriate production process for each vector has been developed. When the manipulated or recombinant vectors are injected into the blood stream, they (by manipulating their tropism) target the liver cells and deliver their genome carrying the FVIII or FIX transgene into the cells. Consequently, the targeted liver cells will use the newly acquired transgenes for the synthesis of the respective protein.

Based on viral vectors, many gene experiments have been carried out in animal models with partially long-lasting gene expression (table 1). In general, gene therapy experiments on smaller models (mice) did show better expression results than studies on larger animals (canine). However, gene therapeutically expressed FIX showed higher activity and persisted longer than FVIII. Based on these results, 5 different clinical studies comprising around 40 hemophilia A/B patients have been started (table 2). In 4 cases, viral vectors (adeno-associated virus, adenovirus, retrovirus) were used, and in 1 case, patients received an expression plasmid. The results were dis-

Table 2. Results of clinical protocols in hemophilia A/B patients treated with transfer vectors containing the FIX or the FVIII gene

Number of patients	Gene transfer system	FIX:C	Expression time, months	Side effects
6	adeno-associated virus (liver) [7]	3–12% (2 patients)	1.5	virus in semen
9	adeno-associated virus (i.m.) [7]	3–4% (2 patients)	5	–
		FVIII:C, %		
13	retrovirus (i.v.) [8]	1% (6 patients)	> 4	–
3	adenovirus (i.v.) [9]	1% (3 patients)	several	↑ trans/thromb↓
12	plasmid10 [10]	1–2% (6 patients)	12	–

i.m. = Intramuscular; i.v. = intravenous.

↑ trans = High transaminase values; thromb↓: low thrombocyte count.

Fig. 1. Regulation of human factor IX-expression by doxycycline (Dox) in vivo in mice: **a** measurement of FIX antigen and **b** FIX activity. SCID mice were injected with 3×10^{10} virus particles per mouse of each of regulator and response vector. Six mice continuously received water with Dox (+), 6 mice repetitively received water with or without Dox (+/-) and another 6 mice received water without Dox (-). The control group (4 mice) received neither vectors nor Dox. The levels of human FIX antigen (hFIX:Ag) and FIX activity (FIX:C) were measured on days 2, 6, 10, 14, 18, 24, 31, 38, 45, 52 and 60 after infection. hFIX:Ag was not detectable in all control mice (no vectors, no Dox) by ELISA. Basic mouse FIX:C was $104 \pm 13\%$ (range 80–130%) over the experimental period as presented by a double dotted line.

appointing as regards both the expression levels and the periods of expression. Additionally, 2 clinical protocols showed a number of side effects. Hemophilia B patients treated with modified adeno-associated virus showed viral DNA sequences in their semen but not in the sperm cells themselves. Some of the hemophilia A patients treated with a modified adenovirus showed fever, elevated transaminase levels and a drop in platelets count. Consequently, all clinical studies had to be discontinued for the time being.

Despite these setbacks, animal studies have demonstrated that FIX expression can be extremely high with up to 20- to 70-fold increases [1, 11]. However, high doses of expressed human FIX may cause thrombosis in humans [12]. Thus, in future, effective expression of human FIX in humans amenable for either up- or down-regulation of transgene expression would be desirable in gene therapy protocols in order to maintain transgene expression within the therapeutic range for safety reasons.

Material and Methods

To demonstrate the regulation of human FIX, we have developed a doxycycline-inducible expression system for human FIX using adenoviral vectors [13]. For this, we constructed and reproduced 2 different adenoviral vectors: a 'regulator' vector, which contains the activator protein gene, and a 'response' vector, which contains the human FIX gene. Within cells, the activator protein is continuously expressed by the 'regulator' vector. If this activator protein comes in contact with doxycycline, a complex is formed which binds at the starting point (promoter) of the human FIX gene. Now, expression of the human FIX protein can begin. However, if doxycycline is removed, the activator protein detaches from the promoter of the human FIX transgene and expression of human FIX stops. This system is reversible. If doxycycline is added again, the doxycycline activator protein complex activates the FIX synthesis again.

After reproduction of both adenoviral vectors by a special packaging system, we injected these vectors into mice (3×10^{10} virus particles per mouse of each vector system) via the tail vein. One group of mice were supplied with doxycycline in drinking water over the whole time (60 days) of experiment (+Dox), the second group was repetitively induced with doxycycline (+/-Dox), while the third group received no doxycycline (-Dox). The control group of mice received neither vectors nor doxycycline.

Results and Discussion

Results of expressed human FIX antigen as well as FIX activity are shown in figure 1. In the mice continuously induced with doxycycline, the level of human FIX antigen (fig. 1a) peaked 10 days after infection (18.9 µg/ml, 3.8-fold above normal human plasma levels) and showed a slow and gradual decrease over the course of the experiment, which remained within the therapeutic range until day 60 (1.33 µg/ml, about 26.7% of the normal human plasma level). In mice that had been subjected to 6 courses of doxycycline treatment and withdrawal, the level of human FIX expression was up- and down-regulated repeatedly for 2 months. During the entire course of the experiment and at each time point, doxycycline-induced human FIX antigen levels reached values which were either similar (on days 2 and 10) or approximately twice as high (on days 18, 31, 45 and 60) than those observed in mice undergoing continuous doxycycline induction. Mice that had been treated with adenoviral vectors but not induced with doxycycline, showed very low human FIX antigen levels (5–25 ng/ml) during the entire experiment. Control mice did not show any human FIX antigen.

As shown in figure 1b, the activity of human FIX in mice treated continuously and repetitively with doxycycline showed similar induction levels to those observed with human FIX antigen. The activity of human FIX in mice that were not in-

duced with doxycycline ranged from 115–138% over the period of the experiment. This value is slightly above the basal level observed in control mice that received neither doxycycline nor vectors (range 80–130%, mean $\pm$ SD 104 $\pm$ 13%).

Based on the above mentioned data, we addressed the question of whether such high expression levels of FIX could affect the blood procoagulant status in mice.

If increased procoagulant activity exists, the level of D-dimer is elevated. The D-dimer is generated during proteolytic degradation (fibrinolysis) of cross-linked fibrin [14–16]. It has already been shown to be associated with deep vein thrombosis and venous thromboembolism as well as other clinical conditions [14–19]. Our results showed that mice treated with adenoviral vectors expressing human FIX and induced with doxycycline had significantly increased D-dimer levels compared to control mice on both day 10 and 60 after infection, despite the decrease in human FIX expression on day 60 after infection (data not shown). However, there was no significant increase in D-dimer in mice treated with adenoviral vectors expressing FIX but not induced with doxycycline as well as in mice treated with adenoviral vectors expressing the marker protein luciferase (induced with or without doxycycline). These results indicate that high expression levels of human FIX increase the blood procoagulant activity in animals (mice) while adenoviral vectors or doxycycline alone or in combination do not.

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