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Hepatocyte clearance and pharmacokinetics of recombinant factor IX glycosylation variants

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ABSTRACT

Addition of N-linked glycosylation sites has been shown to increase serum half-life and decrease clearance for proteins such as recombinant erythropoietin (EPO). However, factor IX (FIX) variants with additional N-linked glycans ("HG" variants) that were expressed in HKB11 cells showed increased clearance in rat *in vivo* pharmacokinetic studies relative to FIX variants with no additional glycans. Variants with multiple additional glycans were the most rapidly cleared. A rat hepatocyte clearance assay was developed to measure intrinsic clearance of these FIX variants *in vitro*. The rank order of clearance of the variants was the same both *in vivo* and in the *in vitro* hepatocyte assay. In the *in vitro* assay, heparin, galactose, and asialo-orosomucoid inhibited clearance of a FIX HG variant by hepatocytes, and asialo-FIX was rapidly cleared, suggesting roles for the asialoglycoprotein receptor (ASGPR) and cell surface proteoglycans in FIX clearance. Thus the *in vitro* hepatocyte intrinsic clearance assay is both useful and predictive for identifying rapidly cleared recombinant proteins and for helping to identify receptors involved in clearance of proteins by the liver.

1. Introduction

Hemophilia B is a bleeding disorder resulting from an inherited deficiency in coagulation factor IX (FIX). Replacement therapy of FIX via intravenous injection restores normal coagulation to patients with hemophilia B. However, because of the pharmacokinetic (PK) properties of FIX, patients must be injected two or three times per week to keep the level of FIX above the minimal effective concentration. An engineered FIX, with reduced clearance and longer serum half-life, would allow for less frequent dosing and would result in a significant improvement in quality of life for these patients.

To develop a longer-acting FIX protein, we used site-directed mutagenesis to add putative N-linked glycosylation sites to the sequence, in the form of Asn-X-Ser/Thr motifs [1]. Addition of N-linked glycosylation sites has been shown to increase circulating half-life for proteins such as recombinant human growth hormone and erythropoietin (EPO). The EPO analog darbepoetin alfa, which has two additional carbohydrate chains, has a threefold longer serum half-life than recombinant human EPO [2]. Recently, a FIX variant with four additional N-glycosylation sites has been described [3]. Clearance of this variant in mice was reduced fivefold relative to wild type FIX.

In the present study, FIX glycosylation variants were expressed in the human cell line HKB11, which is derived from a fusion between HEK293 cells and a B-cell lymphoma and which we have successfully used for expression of coagulation factor VIII (FVIII). FVIII expression in HKB11 cells was 8- to 30-fold higher than in HEK293 or BHK21 cells [4]. Wild-type FIX has two N-linked glycosylation sites in the activation peptide at positions Asn157 and Asn167. We added up to three additional glycosylation sites to create hyperglycosylated (HG) FIX variants [5].

Variants that retained FIX catalytic activity and contained additional glycan chains were tested for improved PK *in vivo* in rats. Unlike EPO, addition of glycosylation sites to FIX expressed in HKB11 cells resulted in increased clearance *in vivo*. Because the liver is the organ primarily responsible for clearance of coagulation factors from circulation, we developed a hepatocyte clearance assay to measure intrinsic clearance (CL_{int}) of FIX and related proteins *in vitro*. We also evaluated whether clearance of FIX HG variants by hepatocytes could be competitively inhibited with heparin and with ligands for the asialoglycoprotein receptor (ASGPR). This *in vitro* model was also used to determine the effect of enzymatic removal of sialic acid on FIX clearance by hepatocytes.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (catalog number A7030), carbonate/bicarbonate buffer pH 9.5 (cat. no. C3041), galactose, EDTA, Tween 20, orosomucoid (alpha1-acid glycoprotein), neuraminidase-aga-

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rose, vitamin K3, trypan blue, and H_2SO_4 were from Sigma to Aldrich (St. Louis, MO). Heparin was from Baxter (Deerfield, IL). BeneFIX® was from Wyeth Laboratories (Philadelphia, PA). Mononine® was from CSL Behring (Kankakee, IL). Hepatocytes, incubation medium and PBS, pH 7.2 (Ca-free, Mg-free) were from Invitrogen/Life Technologies (Carlsbad, CA).

2.2. Plasmid construction

Plasmid construction for the HG mutants was performed as previously described [5].

2.3. Cell culture

HKB11 cells were cultured in proprietary medium as previously described [4].

BHK21 suspension cells were cultured as previously described [5].

Expression of factor IX was performed as previously described [5]. HKB11 cells were transfected using Lipofectamine® 2000 (Invitrogen/Life Technologies, Grand Island, NY) according to the manufacturer's instructions.

Purification of factor IX proteins was performed as previously described [5,6].

2.4. Pharmacokinetics

Male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN), approximately 10 weeks old, were dosed intravenously at 0.70 mg/kg (bolus injection) with FIX variants ($n = 4$). Blood samples were collected automatically through the jugular vein via Culex® (BASI, Lafayette, IN) at time points from 5 min to 48 h. Sodium citrate 5% was used as the anticoagulant in the ratio 1:9 parts of blood. Plasma was collected after centrifugation at 6000 rpm for 10 min in an Eppendorf (Hamburg, Germany) centrifuge. Plasma clearance (CL) was calculated using the noncompartmental analysis method from the program WinNonLin version 5.2 (Pharsight Corporation, Sunnyvale, CA).

2.5. Preparation of hepatocytes

Cryopreserved primary rat hepatocytes were obtained from Invitrogen/Life Technologies. Each vial (approximately 5 million cells per vial) was thawed, and the cells were added to 10 ml of thawing medium (Invitrogen CM3000 Thawing/Plating Supplement Pack added to 500 ml Williams E Medium), followed by centrifugation at 60*g* for 3 min. The cells were resuspended in incubation medium (Invitrogen CM4000 Cell Maintenance Supplement Pack added to 500 ml Williams E Medium) +0.25% bovine serum albumin (BSA; about 4 ml), and the cells were counted using a hemacytometer. Viable cells were counted after staining with Trypan blue to identify dead cells. Cell viability was 80–82%. Cells were used in the clearance assay immediately after counting.

2.6. In Vitro hepatocyte clearance assay

Primary rat hepatocytes, 1 million viable cells/ml, were incubated with 25 ng/ml of various FIX variants in Invitrogen incubation medium +0.25% BSA, in Eppendorf tubes with gentle end-over-end mixing at 37 °C in a starting volume of 1.2 ml. At each of the indicated time points, 0.25 ml of the mixture was removed and immediately centrifuged to pellet the cells (1000 rpm, 3 min in Eppendorf centrifuge). (Typical time points were 5, 10, 20, 30, 60, 120 min.) Then 0.18 ml of the clarified supernatant was removed, quick frozen, and stored overnight at −80 °C. On the next day, FIX in the supernatants was quantified using an enzyme-linked immunosorbent assay (ELISA) in which the corresponding purified variant protein was used as the standard. No-cell control supernatants in which FIX variants were incubated for 2 h at 37 °C in medium alone were used as the zero time point values. Each incubation was done in triplicate.

Studies in which known receptor ligands were added (Fig. 3) were done using the rapidly cleared HG3/5/9 form of factor IX (25 ng/ml) incubated for one hour as described above, with and without 10 mM D-galactose, 50 mM sodium EDTA, or 200-fold molar excess of orosomucoid (OR), asialo-orosomucoid (ASOR), or heparin. *P* values were calculated using paired *T* test.

Intrinsic clearance (CL_{int}) values were calculated based on the method of Lu et al. [7] using the following equation:

CL_{int} = 0.693/*in vitro* half-life, normalized for the incubation volume and the number of cells. *In vitro* half-life was calculated using the program WinNonLin.

2.7. Preparation of asialo-orosomucoid and asialo-factor IX

Orosomucoid (OR; alpha1-acid glycoprotein, Sigma–Aldrich G9885) or R338A FIX (expressed in BHK21 cells) were de-sialylated by treatment with neuraminidase-agarose. Each protein was first dialysed exhaustively against 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM $CaCl_2$, 50 mM NaCl, pH 6. After dialysis, each protein (0.5 ml of 0.33 mg/ml) was incubated with 0.25 U of neuraminidase-agarose beads (Sigma–Aldrich N5254) for 48 h at room temperature (21 °C). At the end of the 48-h incubation, the beads were removed by centrifugation (Eppendorf centrifuge, 1000 rpm for 3 min).

2.8. Factor IX ELISA

Factor IX quantification in pharmacokinetics studies was performed using ELISA as previously described [5].

Supernatants from hepatocyte incubations were assayed using the same ELISA format, except as follows. Each hepatocyte supernatant was diluted 2-fold in Invitrogen incubation medium +0.25% BSA, and then 0.10 ml of each diluted supernatant was added in triplicate to the ELISA plate. Standards were made from the corresponding purified FIX variant diluted in incubation medium +0.25% BSA, in the range of 50–0.8 ng/ml final concentration.

3. Results

3.1. Comparison of in vivo pharmacokinetics of FIX glycosylation variants to in vitro hepatocyte intrinsic clearance assay

All of the FIX variants that we expressed in HKB11 or BHK21 cells contained a mutation at arginine 338 to alanine (R338A) that showed approximately 4-fold enhanced activity in both chromogenic and activated partial thromboplastin time (aPTT) activity assays [8]. Some variants also carried a mutation at Valine 86 to alanine (V86A) that conferred an additional twofold increase in activity [9]. Neither of these activity-enhancing mutations significantly altered the PK of FIX and did not alter its glycosylation profile (data not shown).

Table 1 shows the putative N-linked glycosylation sites that were added to the FIX sequence using site-directed mutagenesis. Amino acid substitutions were made to create N-X-S/T sequences, which have been shown to be sites of potential N-linked glycosylation [1]. The added sites were numbered HG1 and up. The HG8 modification was an insertion of nine amino acid residues (NSTQDNITQ) between Ala161 and Glu162 that included two potential glycosylation sites. Some variants contained sites that were added in