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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

combination (e.g., HG3/5/9 indicating the presence of the HG3, HG5 and HG9 mutations).

Pharmacokinetic profiles in rats dosed at 0.70 mg/kg (intravenous bolus) of five representative variants are shown in Fig. 1, with calculated clearance (CL) values shown in Table 2. FIX typically exhibits a biphasic PK profile. Three of the variants (HG9, HG3/9/10, and HG3/5/9) with added glycosylation sites were cleared very rapidly compared with the parental R338A FIX, plasma-derived FIX (Mononine®), or Chinese hamster ovary (CHO)–cell expressed FIX (BeneFIX®). These HG variants were also cleared more efficiently in the *in vitro* hepatocyte assay (Fig. 2). The rank order of clearance of the HKB11-expressed variants was the same in both *in vivo* and *in vitro* experiments, as shown in Table 2, which compares *in vivo* CL and *in vitro* CL_{int} values for 12 variants. The *in vitro* intrinsic clearance (CL_{int}) was calculated based on the method of Lu et al. for small molecules (see Section 2) [7]. Although there was not a strong linear correlation between *in vivo* and *in vitro* values, the rank order of clearance of the HG variants was similar, that is, more rapidly cleared forms had the highest CL values in both *in vivo* and *in vitro* measurements. Thus the hepatocyte intrinsic clearance assay was shown to be roughly predictive of the *in vivo* PK of the HG variants.

Hepatocytes have widely been used to measure clearance of small molecule drugs [10–12]. However, they have not often been used to study clearance of proteins. The *in vitro* clearance assay method developed in this study used cryopreserved primary rat hepatocytes that were thawed, suspended and immediately incubated with FIX in tubes that were gently rotated end over end. We chose cryopreserved hepatocytes because published studies showed that their performance was comparable to that of freshly isolated hepatocytes (at least for small molecule clearance) [10,13,14], and vials of cryopreserved hepatocytes from the same pooled cells could be individually thawed over a period of days or weeks, yielding consistent results. We obtained similar results between cryopreserved rat hepatocytes plated in 24 well plates compared with suspension cultures in Eppendorf tubes. Primary human hepatocytes in suspension also cleared HG variants in the same rank order, but less efficiently, than rat hepatocytes (data not shown).

Comparison of the *in vivo* CL for variants expressed in two different cell lines (HKB11 and BHK21) shows that in each case clearance was higher for the HKB11-expressed proteins relative to their BHK21-expressed counterparts (Table 2). The *in vitro* clearance assay was not able to distinguish between HKB11-expressed molecules that showed *in vivo* CL values of about 100 ml/h/kg and

Table 1
Sequence changes resulting in addition of putative glycosylation sites.

Variant name	Sequence changes
HG3	P151N, V153T
HG5	T172N, S174T
HG8	Insertion of 9aa between A161 and E162 (NSTQDNITQ)
HG9	G226N, K228T
HG10	D85N

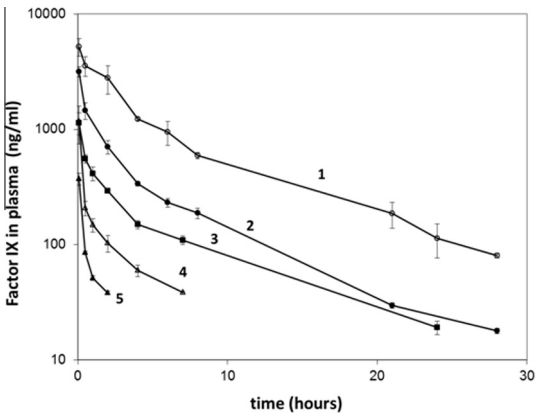

Fig. 1. Pharmacokinetics of FIX variants in rats. Rats were intravenously injected with 0.70 mg/kg of CHO cell–expressed FIX (BeneFIX®), HKB11 cell–expressed R338A FIX (no added HG sites), or HKB11 cell–expressed glycosylation variants (HG9, HG3/9/10, HG3/5/9). Blood samples were drawn at various time points using Culex™ (BASI, Lafayette, IN). FIX antigen was quantified using ELISA. Data are presented as mean ± SD ($n = 4$). Key: CHO cell expressed FIX (BeneFIX®, 1, open circles), HKB11 cell expressed R338A FIX (no added HG sites, 2, solid circles) or HKB11 cell expressed glycosylation variants (HG9, 3, squares; HG3/9/10, 4, open triangles; HG3/5/9, 5, solid triangles). CHO = Chinese hamster ovary; ELISA = enzyme-linked immunosorbent assay; FIX = factor IX.

Table 2
In vitro and *in vivo* clearance of FIX variants.

Molecule	Cell line	*In vitro*	*In vivo*
		CL_{int} (uL/min/ml cells)	CL ml/h/kg
Mononine® (wild type)	Plasma	0.8	45.0
Benefix® (wild type)	CHO	0.6	34.8
R338A	HKB11	0.9	116.0
R338AV86A	HKB11	1.8	100.0
R338AV86A-HG3/8	HKB11	2.1	109.0
R338A HG3	HKB11	2.6	135.0
R338A HG9	HKB11	4.6	241.0
R338AV86A HG3/9/10	HKB11	10.1	662.0
R338A HG3/5/9	HKB11	12.3	2853.0
R338A	BHK21	1.2	45.3
R338AV86A	BHK21	0.7	32.0
R338AV86A-HG3/8	BHK21	1.0	12.8
R338A HG3	BHK21	1.1	16.1
R338A HG3/5/9	BHK21	ND	11.0

CHO = Chinese hamster ovary cells; CL = clearance; CL_{int} = intrinsic clearance; ND = no data (all of the protein was consumed in the *in vivo* study).

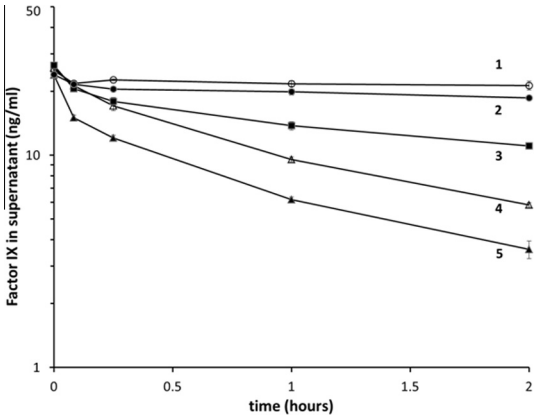

Fig. 2. *In vitro* hepatocyte clearance assay. Primary rat hepatocytes, 1 million viable cells/ml, were incubated with 25 ng/ml of various FIX variants with mixing at 37 °C in a starting volume of 1.2 ml. At each of the indicated time points, 0.25 ml of the mixture was removed and immediately centrifuged to pellet the cells. Then, 0.18 ml of the supernatant was removed, quick frozen, and stored overnight. On the next day, the FIX in all of the supernatants was quantified using ELISA, in which the corresponding purified variant protein was used as the standard. Controls in which FIX variants were incubated for 2 h without cells were used as the zero time point values. Each incubation was performed in triplicate. Data are presented as mean ± SD and are representative of at least 3 independent experiments. Key: CHO cell expressed FIX (BeneFIX®, 1, open circles), HKB11 cell expressed R338A FIX (no added HG sites, 2, solid circles) or HKB11 cell expressed glycosylation variants (HG9, 3, squares; HG3/9/10, 4, open triangles; HG3/5/9, 5, solid triangles). CHO = Chinese hamster ovary; ELISA = enzyme-linked immunosorbent assay; FIX = factor IX.

their BHK21-expressed counterparts that showed CL values of 10–30 ml/h/kg (*in vitro* CL_{int} values were 0.7–2.1 ml/h/kg for all of them). However, the assay easily identified the rapidly cleared HKB11-expressed variants HG9, HG3/9/10 and HG3/5/9. The variant that showed the most rapid clearance, HKB11-expressed HG3/5/9, showed much reduced clearance *in vivo* when expressed in BHK21 cells (Table 2). (The BHK21-expressed variant was not tested in the hepatocyte clearance assay because all of the available protein was consumed in the *in vivo* study). We subsequently used BHK21 cells to express additional variants with multiple HG sites that exhibited even lower *in vivo* clearance than the variants discussed in the present study [5].

In vitro CL_{int} values for our BHK21-expressed FIX variants were similar to those of wild-type FIX derived from human plasma (Mononine®) and to CHO cell–expressed FIX (BeneFIX®; Table 2).

3.2. Competition with receptor ligands; clearance of asialo-FIX

Although FVIII and activated factor VII (FVIIa) have been shown to bind to the hepatic asialoglycoprotein receptor (ASGPR), the receptors involved in FIX clearance have not been elucidated. Using ligands for previously known receptors to compete for coagulation factor binding to hepatocytes, we sought to identify the receptors involved in clearance. We used the hepatocyte clearance assay to study the effects of heparin, galactose or asialo-orosomucoid (ASOR) on the clearance of FIX. ASOR is prepared by desialylating the plasma protein orosomucoid (OR) using neuraminidase-agarose, which cleaves off the terminal sialic acids from the glycans. Clearance of the HG3/5/9 variant was inhibited by the ASGPR ligand ASOR but not by OR (Fig. 3). Clearance was also inhibited by ethylenediaminetetraacetic acid (EDTA) and was partially inhibited by galactose. Heparin partially inhibited binding of the HG3/5/9 variant, indicating a role for cell surface heparan sulfate proteoglycans.

We measured sialic acid content of our variants using matrix-assisted laser desorption ionization (MALDI) mass spectrometry. HKB11 cell–expressed variants had fewer overall sialic acid molecules per glycan site than BHK21-expressed proteins. Those HKB11-expressed variants with three additional sites (HG3/9/10, HG3/5/9) had lower sialic acid content than those with one or two added sites, and these had lower sialic acid content than those variants with no added sites. HG3/5/9, which had the highest clearance, had the lowest sialic acid content per site (data not shown, Richard Feldman, manuscript in progress 2013). The reduced sialylation of the HKB11 cell–expressed FIX proteins also implicates ASGPR as the receptor involved in clearance. To more directly address the role of sialic acid in FIX uptake by hepatocytes, we compared FIX (R338A form expressed in BHK21 cells) to asialo-FIX (desialylated FIX) in the hepatocyte clearance assay. The asialo-R338A FIX was rapidly cleared compared with R338A FIX, suggesting that unsialylated FIX binds efficiently to hepatocytes via ASGPR (Fig. 4).

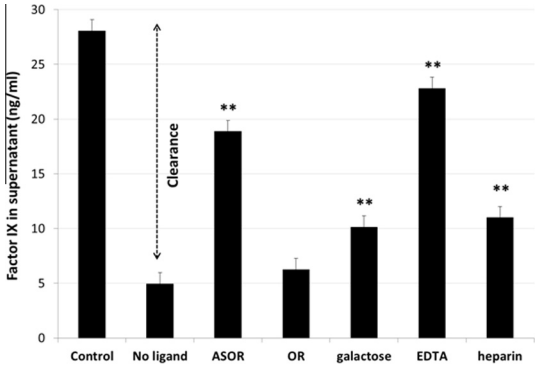

Fig. 3. Effect of receptor ligands and EDTA on clearance of HG3/5/9 FIX by primary rat hepatocytes. HKB11 cell–expressed HG3/5/9 FIX (25 ng/ml) was incubated with primary rat hepatocytes for 1 h at 37 °C as described in the Fig. 2 legend, with or without 10 mM D-galactose, 50 mM sodium EDTA, or 200-fold molar excess of OR, ASOR, or heparin. "Control" represents incubation without hepatocytes. Each incubation was performed in triplicate. Data are presented as mean ± SD. Asterisks indicate a statistically significant difference when comparisons were made to the "no ligand" control: $^{**}P < 0.01$; also, $P < 0.005$ for ASOR compared to OR. ASOR = asialo-orosomucoid; EDTA = ethylenediaminetetraacetic acid; FIX = factor IX; OR = orosomucoid.

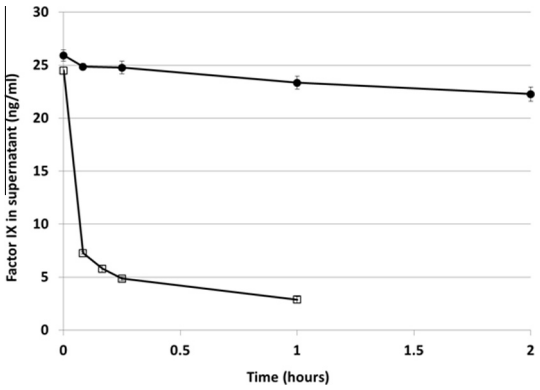

Fig. 4. Clearance of FIX and asialo-FIX (R338A forms) by primary rat hepatocytes. BHK21 cell–expressed R338A FIX (solid circles) or asialo-FIX(desialylated FIX), prepared by treatment of R338A FIX with neuraminidase (open squares), was incubated at 25 ng/ml with primary rat hepatocytes for 1 h at 37 °C as described in the Fig. 2 legend. Each incubation was performed in triplicate. Data are presented as mean ± SD. FIX = factor IX.

4. Discussion

Clearance of FIX variants containing added glycosylation sites was increased for HKB11-expressed proteins, with variants having three added sites showing the most rapid clearance. Variants expressed in BHK21 cells, however, did not show increased clearance with addition of glycosylation sites. The HKB11 cell line was selected because of its rapid growth and high protein expression level, with the advantage of rapid expression of a large number of FIX variant proteins for PK studies. The rapid clearance of the HKB11-expressed HG variants indicates that the choice of cell line for expression of recombinant proteins is critical.

Coagulation factors have been observed to be cleared by the liver. FVIII has been shown to be cleared in mice via the ASGPR, interacting with the receptor through its B domain. ASGPR binding and *in vivo* clearance of FVIII was inhibited by the addition of the ASGPR antagonist ASOR, by EDTA, and by D-galactose [15]. FVIII has also been shown to bind to the low-density-lipoprotein receptor-related protein (LRP), and that binding was facilitated by cell surface proteoglycans and could be inhibited by addition of heparin [16].

Factor VII, which is structurally similar to FIX but has a much shorter half-life than native FIX, was shown to bind to primary rat hepatocytes *in vitro* [17]. Asialo-FVIIa hepatocyte binding and internalization was similar to that of FVIIa, leading to the conclusion that FVIIa binding to hepatocytes is not via the ASGPR [17].

However, data from *in vivo* rat PK studies and a perfused liver model showed increased clearance of asialo-FVIIa relative to FVIIa and inhibition by ASOR [18]. This contrast between studies suggests that types of liver cells other than hepatocytes may be involved in clearance of FVIIa, although hepatocytes make up 80–90% of liver cells [19]. For FIX, hepatocytes appear to be the primary cell

type involved in clearance in rats because the *in vitro* hepatocyte clearance assay was predictive of *in vivo* PK data. FIX has also been shown to bind to type IV collagen on endothelial cells [20], however, endothelial cell binding appears not to be the primary clearance pathway for rapidly cleared forms of FIX.

Although the output of our *in vitro* hepatocyte assay is clearance, the assay indirectly measures binding of FIX to receptors or other molecules on the cell surface. Using the assay, we studied whether the binding of FIX to hepatocytes could be competed with EDTA, galactose or ASOR. All of these reagents have been shown to inhibit binding of FVIII to the ASGPR as discussed previously. Intrinsic clearance of the HG3/5/9 variant was inhibited by ASOR but not by orosomucoid (OR). Clearance was partially inhibited by galactose, which competes for binding of ASGPR to exposed galactose residues on unsialylated glycans. ASGPR binding of FVIII is calcium-dependent [15]. The observation that EDTA blocked FIX clearance in our hepatocyte assay suggests that FIX binding to ASGPR is also calcium dependent.

Whether or not ASGPR is the primary receptor for clearance of native glycoproteins is controversial [21]. However, our data clearly show that ASGPR is involved in clearance of poorly sialylated forms. The fact that our HKB11-expressed variants with added HG sites have lower sialic acid content than BHK21 cell–expressed variants supports this conclusion. Further support comes from the observation that HKB11-expressed variants with three added sites, which are cleared the most rapidly, have fewer sialic acids per site than those variants with zero, one, or two additional glycosylation sites. A recent study of FVIIa binding to hepatocytes suggests that ASGPR is involved in clearance of unsialylated FVIIa, which comprises about 20% of circulating FVIIa [19]. ASGPR is likely to function as a receptor for clearing unsialylated FIX as well. Binding of the FVIII-vWF complex to cell surface proteoglycans facilitates binding to the LRP, and this binding is inhibited by heparin [16]. In our *in vitro* clearance assay, heparin partially inhibited binding of the HG3/5/9 variant to hepatocytes, suggesting that FIX also binds to cell surface proteoglycans.

In conclusion, the *in vitro* hepatocyte clearance assay is an effective tool for identifying rapidly cleared recombinant FIX protein variants. The assay could be applied to other proteins that are cleared by the liver, allowing researchers to identify rapidly cleared forms and thus minimize the number of costly *in vivo* PK studies. The assay also requires much less protein than an *in vivo* study. Furthermore, the assay can be useful for helping to identify receptors involved in clearance of proteins by the liver. Our data suggest that the receptor ASGPR plays a role in clearance of unsialylated FIX, as has been postulated for factor VII.

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