Characterization of Post-translational Modifications and Resulting Structure/Function Relationships of Recombinant Human Factor IX Produced in the Milk of Transgenic Pigs

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(Abstract)

Hemophilia B is a debilitating and life-threatening disorder caused by a deficiency in or dysfunction of factor IX (FIX), a complex plasma glycoprotein required for the formation and maintenance of blood clots. Treatment of hemophilia B involves infusion of replacement FIX currently derived from two sources: FIX purified from pools of human plasma (pd-FIX) and a single recombinant FIX product generated in genetically engineered Chinese hamster ovary (CHO) cells. Both of these FIX products are prohibitively expensive, limiting of the treatment options of hemophiliacs worldwide. As a result, a more abundant and affordable FIX product would greatly improve the life prospects for hemophiliacs.

The biological activity of FIX is dependent upon its numerous post-translational modifications (PTMs), including γ-carboxylation, proteolytic maturation, phosphorylation, sulfation, and glycosylation. Of these PTMs, those known to be vital for activity are γ-carboxylation of multiple glutamate residues near the N-terminus and proteolytic cleavage of the FIX propeptide. When expressed at a high rate in exogenous expression systems, however, the ability of current systems to effect the necessary PTMs is severely rate limited, restricting the production of active FIX.

The transgenic pig bioreactor represents a promising source for the production of large quantities biologically active FIX due to its demonstrated ability to perform the required FIX PTMs. It was the goal of this study to characterize the PTM structure and the resulting function of recombinant FIX when expressed at 1-3 mg/ml in the transgenic pig mammary epithelium (tg-FIX). It was found that the expressed tg-FIX is comprised of a heterogeneous mixture of FIX PTM isoforms. This mixture represents a spectrum of tg-FIX molecules of varying γ-carboxyglutamic acid (Gla) and propeptide content, indicating that rate limitations in effecting these PTMs are present. A purification process was developed utilizing heparin-affinity chromatography to purify the total population of tg-FIX from pig milk, a complex multi-phase feedstock. Subsequently, a process was developed to fractionate the total population of tg-FIX into subpopulations based upon the extent of post-translational modification. Q ion-exchange chromatography was utilized to fractionate tg-FIX based upon molecular acidity which was found to be correlated to both biological activity and Gla content. The resulting biologically active tg-FIX population contained an average of 7 of the 12 Gla residues found in pd-FIX. Immuno-affinity chromatography was subsequently utilized to further fractionate tg-FIX into mature tg-FIX and propeptide-containing tg-FIX populations.

The isolated FIX PTM populations were subjected to functional analysis by investigating *in vitro* clotting activity, activation by factor XIa, and *in vivo* pharmacokinetics. From this analysis it was found that mature tg-FIX with an average 7 Gla residues, representing approximately 9% of the total tg-FIX produced, exhibits wild-

type *in vitro* clotting activity and normal activation by factor XIa. The remainder of the tg-FIX produced, characterized by either a lower Gla content or the presence of the propeptide, was found to be inactive and displayed less efficient activation by factor IXa. In an *in vivo* pharmacokinetic study in the hemophilia B mouse model, biologically active tg-FIX was found to possess altered circulating properties. Tg-FIX was characterized by a lower recovery, approximately one-sixth that of pd-FIX, but an extended circulation half-life. From this study it was found that the mean residence time of tg-FIX after injections is approximately twice that observed for pd-FIX. These altered pharmacokinetic properties are likely linked to the unique tg-FIX PTM structure, perhaps through altered endothelial cell binding characteristics caused by the reduced Gla content.

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List of Amino Acid Abbreviations

Amino Acid	Three Letter Abbreviation	One Letter Abbreviation
Alanine	Ala	A
Cysteine	Cys	C
Aspartate	Asp	D
Glutamate	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y