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Development of a lectin binding assay to differentiate between recombinant and endogenous proteins in pharmacokinetic studies of protein-biopharmaceuticals

Alfred Weber[a], Eva Minibeck[a], Friedrich Scheiflinger[b], Peter L. Turecek[b,*]

[a] Baxter Innovations GmbH, Benatzkygasse 2-6, 1220 Vienna, Austria
[b] Baxter Innovations GmbH, DC Tower, Donau-City-Straße 7, 1220 Vienna, Austria

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ABSTRACT

Human glycoproteins, expressed in hamster cell lines, show similar glycosylation patterns to naturally occurring human molecules except for a minute difference in the linkage of terminal sialic acid: both cell types lack α2,6-galactosyl-sialyltransferase, abundantly expressed in human hepatocytes and responsible for the α2,6-sialylation of circulating glycoproteins. This minute difference, which is currently not known to have any physiological relevance, was the basis for the selective measurement of recombinant glycoproteins in the presence of their endogenous counterparts. The assay is based on using the lectin *Sambucus nigra* agglutinin (SNA), selectively binding to α2,6-sialylated *N*-glycans. Using von Willebrand factor (VWF), factor IX (FIX), and factor VIIa (FVIIa), it was demonstrated that (i) the plasma-derived proteins, but not the corresponding recombinant proteins, specifically bind to SNA and (ii) this binding can be used to deplete the plasma-derived proteins. The feasibility of this approach was confirmed in spike-recovery studies for all three recombinant coagulation proteins in human plasma and for recombinant VWF (rVWF) in macaque plasma. Analysis of plasma samples from macaques after administration of recombinant and a plasma-derived VWF demonstrated the suitability and robustness of this approach. Data showed that rVWF could be selectively measured without changing the ELISAs and furthermore revealed the limitations of baseline adjustment using a single measurement of the predose concentration only. The SNA gel-based depletion procedure can easily be integrated in existing procedures as a specific sample pre-treatment step. While ELISA-based methods were used to measure the recombinant coagulation proteins in the supernatants obtained by depletion, this procedure is applicable for all biochemical analyses.

1. Introduction

Glycosylation is by far the most important and complex post-translational protein modification. It supports proper folding, enables targeting of protein and ligand binding, modulates biological activity, contributes to stability and determines circulatory serum half-lives [1–5]. The carbohydrate to the protein linkage allows differentiation between different classes. *N*-Glycans were discovered early as they are abundant components of plasma proteins [6] with the first reports on the nature of the linkage published for IgG, α_1-acid glycoprotein, and transferrin in the early 1960s. These N-glycans share a complex biosynthetic pathway in which a lipid-bound oligosaccharide is transferred by the oligosaccharyltransferase to acceptor "sequons" on nascent proteins. These sequons are formed by the sequence Asn-X-Ser/Thr, where X is any amino acid except Pro. Along its secretory pathway from the ER to the Golgi, this oligosaccharide is then processed by various glycosidases and glycosyltransferases, finally giving rise to oligomannosidic, hybrid, and complex *N*-glycans. Complex *N*-glycans, composed of the monosaccharides N-acetylglucosamine, mannose, galactose, fucose, and N-acetylneuraminic acid (NeuAc), predominate on circulatory plasma glycoproteins. Plasma glycoproteins can carry multiple *N*-glycans on their protein backbone with site-specific structures as described for the three *N*-glycans on human α_1-antitrypsin [7], or randomly distributed as reported for the nine *N*-glycans on human buturylcholinesterase [8]. The terminal

* Corresponding author. Tel.: +43 1 20100 247 2520; fax: +43 20100 247 5733.
E-mail addresses: alfred_weber@baxter.com (A. Weber), eva_minibeck@baxter.com (E. Minibeck), fritz_scheiflinger@baxter.com (F. Scheiflinger), peter_turecek@baxter.com (P.L. Turecek).

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glycosylation pattern of human plasma glycoproteins, however, is relatively uniform as most oligosaccharides are capped with at least one NeuAc attached to galactose in α2,3- or α2,6-linkage.

Although human glycoproteins seem to contain less α2,6- than α2,3-bound NeuAc, hepatocytes express relatively high levels of the sialyltransferase ST6Gal-I. This gives rise to the predominant α2,6-sialylation of plasma glycoproteins. The lectin SNA, being specific for α2,6-linked NeuAc [9], binds to the majority of rat plasma proteins. More specifically, α2,6-bound NeuAc has been detected in many abundant human plasma glycoproteins including fibrinogen [10], transferrin, α_2-macroglobulin, immunoglobulins, haptoglobin, orosomucoid, C1-inhibitor, and α_1-antitrypsin [7].

The multiple essential functions associated with the carbohydrate moieties of glycoproteins, the fact that almost half of therapeutically used proteins are glycosylated, and the occurrence of human natural antibodies against non-human xenogeneic glycostructures [11,12] have contributed to the development and selection of appropriate expression systems for recombinant protein production. To date, two hamster cell lines, baby hamster kidney (BHK) and Chinese hamster ovary (CHO), are widely used for large-scale expression of therapeutic proteins. Both cells provide glycosylation patterns similar to those on human plasma proteins except for a minute difference in the N-glycan sialylation: both cells lack the sialyltransferase ST6Gal-I responsible for α2,6-sialylation of *N*-glycans [13]. Thus, all complex *N*-glycans, although extensively sialylated, terminate with α2,3-linked NeuAc.

Treatment of coagulation disorders has markedly improved since recombinant coagulation proteins became available. Thus, recombinant factor VIII (FVIII), full-length and various brands of B-domain-deleted FVIII preparations [14], rFIX [15], and rFVIIa [16] have been approved for hemophilia A and B patients. A recombinant rVWF for treatment of von Willebrand disease is in late clinical development.

Here, the minute difference in the linkage of terminal sialic acid between CHO and BHK cell-derived and human plasma-derived (pd) glycoproteins was utilized to develop a method for selectively measuring these recombinant proteins. This method should facilitate preclinical studies in non-human primates, as such studies are often hampered by the high degree of cross-reactivity between endogenous (animal) and recombinant human protein, making a specific measurement of the recombinant protein impossible.

2. Materials and methods

2.1. Reagents, chemicals and biological materials

The following chemicals were from Sigma (Vienna, Austria): Neuraminidase (N-2133), 6′- and 3′-sialyllactose (A-9204; A-8681), bovine serum albumin (A0281; BSA), benzamidine, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium (meta) periodate and Bis-Tris-Propane. Tween 20 was obtained from Bio-Rad (Vienna Austria), Patentblau V from Chroma-Waldeck (Münster, Germany), the peroxidase substrate SureBlue from KPL (Hamburg, Germany), skimmed milk (Fixmilch Instant) was from Maresi (Vienna, Austria). All other chemicals and 96-well Maxisorp F96 plates were from VWR (Vienna, Austria). The following biological reagents were used: rabbit anti-human VWF IgG (A0082), rabbit anti-human VWF IgG peroxidase (P0226), rabbit anti-human FIX IgG (A0300) and streptavidin peroxidase (P0397) were from DakoCytomation (Glostrup, Denmark), sheep anti-human FIX (FIX-EIA C, FIX-EIA D) and sheep anti-human FVII (FVII-EIA C, FVII-EIA D) from CoaChrom (Vienna, Austria). Biotinylated SNA (B-1305) and SNA agarose (AL-1303; 3 mg SNA/mL) were purchased from Vector (Peterborough, UK). CNBr-Sepharose (GE Healthcare, Vienna, Austria) was used to couple SNA at a concentration of 6 mg/mL. CHO cell-derived rVWF, rFIX and rFVIIa (Baxter BioScience) and the coagulation factors BeneFIX (Pfizer; rFIX) and NovoSeven (Novo Nordisk; rFVIIa), the latter expressed in BHK cells, were investigated. Human reference plasma preparations were purchased from Technoclone (Vienna, Austria); the human pdVWF concentrate Haemate-P was obtained from CSL Behring. Macaque plasma samples came from a preclinical study performed according the United Kingdom Animals (Scientific Procedures) Act 1986 and associated Codes of Practice for the Housing and Care of Animals used in scientific procedures and were re-analyzed after storage at ≤−60 °C.

2.2. VWF, FIX and FVII/FVIIa ELISA

VWF, FIX, and FVII/FVIIa were measured by ELISA using paired polyclonal antibodies as described [17]. Samples were diluted with phosphate-buffered saline (PBS) containing 2 mM benzamidine and 0.1% Tween 20, including 0.1% skimmed milk for FIX and VWF and 0.1% BSA for FVII/FVIIa. Geometric dilution series of the standards or samples were incubated with the blocked wells at room temperature (RT) using single incubation multilayer immune technique [18] for VWF and FIX ELISA. The calibration curves ranged from 21.8 to 0.68 mU/mL (1.14–0.07 mU/mL in a more sensitive format) for the VWF ELISA, from 27 to 1.7 ng/mL for the FIX ELISA, and from 17.7 to 0.6 ng/mL for FVII/FVIIa ELISA.

2.3. ELISAs to measure α2,6-sialylation

The presence of α2,6-NeuAc was confirmed using a lectin ELISA. The plates were coated according to the respective ELISA protocols and blocked. Unfavorable lectin binding to the capture antibodies was prevented by mild periodate oxidation using 10 mM sodium periodate in 50 mM sodium acetate, pH 5.5 for 30 min followed by incubation with 1% (v/v) ethanolamine solution for 5 min. Incubation was terminated by a washing step. Biotinylated SNA (2 μg/mL), streptavidin peroxidase and SureBlue were sequentially used to detect α2,6-NeuAc on the plate bound proteins. Human reference plasma was used as a source for pd proteins. Linear concentration responses were detected at 2.85–0.18 mU/mL and 6.2–0.19 mU/mL for VWF and FIX, respectively and at 5–0.16 ng/mL for FVII/FVIIa, using a FVII plasma concentration of 500 ng/mL [19] for the conversion of plasma units to mass.

The binding specificity was confirmed by desialylation and by competition with sialyllactose. For desialylation, plate-bound pdVWF (5 and 2.5 mU/mL) was incubated with neuraminidase (0–20 mU/mL) in neuraminidase incubation buffer (20 mM Bis-Tris-Propane, pH 6.0; 2 mg/mL BSA) at 37 °C for 2 h. Treatment effects were detected with biotinylated SNA as described above. For competition, pdVWF (5 mU/mL) was incubated with increasing concentrations of 6′- or 3′-sialyllactose (2.1–150 μM and 16 to 140 μM, respectively), before adding biotinylated SNA as describe above.

2.4. SNA gel-based depletion for endogenous proteins

For mixtures of rVWF with human/macaque pdVWF, the decrease in SNA binding strictly was demonstrated to correlate with relative concentrations of rVWF. However, this approach requires two measurements, and variations in endogenous VWF levels would still affect the results. Alternatively, a depletion of α2,6-sialylated proteins was carried out. The resulting depletion supernatant then allowed the selective measurement of the recombinant protein. Using separation of pdVWF and rVWF as a worst case scenario, as the size of VWF is likely to impede its binding to immobilized SNA, the completeness of pdVWF depletion was verified. 300 μL human plasma, diluted 1/10, 1/20, and 1/50 with depletion buffer (20 mM Tris, 150 mM NaCl, pH 7,4; 5 mg/mL BSA)