Comparison of N-Glycan Pattern of Recombinant Human Coagulation Factors II and IX Expressed in Chinese Hamster Ovary (CHO) and African Green Monkey (Vero) Cells

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Abstract. The N-glycan patterns of recombinant human coagulation factors II (rF-II) and IX (rF-IX), derived from both transfected Chinese hamster ovary (CHO) cells and African green monkey (Vero) cells produced at industrial scale, were analyzed by binding to carbohydrate-specific lectins and were compared with the glycan structure of human plasma-derived coagulation factors. Human plasma-derived coagulation factors II (hpF-II) and IX (hpF-IX) exhibited complex-type glycan structures with carbohydrate chains capped with α(2-6)-sialic acid. Terminal galactose-β(1-4)-N-acetylglucosamine units were detected in hpF-IX. Both CHO cell-derived rF-II and rF-IX exhibited complex-type glycosylation and contained α(2-3)-sialic acid in addition to terminal galactose-β(1-4)-N-acetylglucosamine. Vero cell-derived rF-IX exhibited a complex-type glycan structure similar to that of CHO cell-derived rF-IX. In contrast, rF-II produced by Vero cells exhibited a glycan microheterogeneity composed of hybrid-type glycosylation containing "high-mannose" structures and complex-type glycosylation containing α(2-3)-sialic acid. Galactose-β(1-4)-N-acetylglucosamine structures and a low concentration of α(2-6)-sialic acid were detected in both microheterogeneity fractions of Vero cell-derived rF-II. Although different in their carbohydrate structures, coagulation factors II and IX obtained recombinantly from both transformed CHO cells and Vero cells exhibited coagulation activities comparable with the plasma-derived proteins.

Key Words. coagulation factor, recombinant, glycosylation.

Recombinant technology promises several major benefits for the production of human plasma proteins: the possibility of supplying purer and safer proteins in quantity, little or no contamination with pathogenic materials, and the possibility of creating modified coagulation factors with modified properties [1,2]. However, most human plasma proteins are glycoproteins [3]. Oligosaccharides linked to proteins may contribute to receptor-mediated interactions, protein stability, clearance from circulation, and physiological function of the protein [4-6]. Given the importance of protein glycosylation for pharmacological activity, expression of such molecules in mammalian cells is therefore the one and only choice for the manufacture of correctly glycosylated biopharmaceuticals [7,8].

There is no or little information available, however, about the glycosylation structure of human plasma-derived coagulation factors II and IX isolated at a manufacturing scale from human plasma for use as biopharmaceuticals. Industrial scale productions of recombinant coagulation factors II and IX have not been reported at all. In this study, for the first time the glycan pattern of coagulation factor II (prothrombin) and coagulation factor IX (Christmas factor) produced recombinantly on an industrial scale have been investigated. Both recombinant coagulation factors were obtained from permanently expressing transfected Chinese hamster ovary (CHO) cells and from transient expression of Vaccinia virus–infected African green monkey (Vero) cells, and were compared with the glycan pattern of the corresponding human plasma proteins.

Material and Methods

Materials

Plasma-derived human prothrombin (human coagulation factor II, hpF-II) and plasma-derived human coagulation factor IX (hpF-IX) were from Diagnostica Stago (France). Recombinant coagulation factors were produced from cell suspension culture in 80 l industrial fermentors (Vero cell culture) or from high cell density perfusion culture on macroporous carriers in industrial 10 l fluidized bed reactors (CHO cell culture). Recombinant coagulation factors were isolated from cell culture supernatants by a combination of anion exchange chromatography and calcium-dependent anion exchange filtration, as described in detail previously [9]. N-glycosidase F from F. menin-
Isolation of glycoprotein equilibrated with 50 mM Tris/HCl buffer at pH 7.4, were loaded onto a MONO-Q column (Pharmacia), microheterogeneities. Recombinant proteins in 50 mM Tris/HCl buffer, pH 7.4, resulted in the separation of two glycosylation microheterogeneities. The V/VrF-II fraction resulted in molecular weights of about 68,000 Da and 60,000 Da for both recombinant and plasma-derived coagulation factors II (Figure 2) and IX (Figure 3), respectively. Deglycosylation of recombinant coagulation factors was confirmed by incubation of the proteins with N-glycosidase F to cleave asparagine-bound N-glycans. Deglycosylation resulted in the reduction of the molecular weights of both the plasma-derived and the recombinant coagulation factors, and resulting apparent molecular weights of about 68,000 Da and 60,000 Da for both recombinant and plasma-derived coagulation factors II (Figure 2) and IX (Figure 3), respectively.

For detection of the N-glycosylation pattern, coagulation factor activity was determined by modified prothrombin time test and by a one-stage coagulation assay, respectively, with the coagulumeter KC 10, as described previously [9]. Coagulation factor II and IX concentration standards (Immuno AG) were used as references. One activity unit refers to coagulation factor activity in 1 ml of pooled normal plasma.

Electrophoresis
Electrophoretic analysis of protein was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide slab gels under reducing conditions using the buffer system described by Laemmli [11]. After electrophoresis, proteins in the gel were either silver stained using a commercial kit (Bio-Rad) or electrobotted onto nitrocellulose membranes in 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol buffer [12].

Detection of glycan structures with digoxigenin-labeled lectins
After SDS-PAGE and electrotransfer of proteins onto nitrocellulose membranes, glycoproteins were incubated with digoxigenin-labeled lectins followed by detection of glycoprotein-bound lectins with alkaline phosphatase-conjugated anti-digoxigenin immunoglobulin (Boehringer Mannheim). Fetuin and asialofetuin were used as control glycoproteins to certify the reactivity of the lectins.

Isolation of glycoprotein microheterogeneities
Recombinant proteins in 50 mM Tris/HCl buffer, pH 7.4, were loaded onto a MONO-Q column (Pharmacia), equilibrated with 50 mM Tris/HCl buffer at pH 7.4, and eluted with a linear NaCl gradient of 150 to 500 mM. Fractions containing protein were collected and tested for enzyme activity and protein concentration, and were analyzed by SDS-PAGE followed by electroblotting and glycan detection by specific lectins.

Desialidation of glycoproteins
Proteins (0.2 mg/ml) in 20 mM phosphate buffer, pH 7.0, containing 5 mM EDTA, were incubated with 20 mU sialidase/ml at 37°C for 12 hours. Samples taken prior to addition of sialidase and after incubation were analyzed by SDS-PAGE and lectin binding.

Results
Recombinant human coagulation factors II and IX were purified from both cell culture supernatants of Vaccinia virus–infected Vero cells and cell culture supernatants of transfected Chinese hamster ovary (CHO) cells. Figure 1 shows that Vero/Vaccinia-derived rF-II (V/VrF-II) and CHO-derived r-FII (CHOOrF-II), and Vero/Vaccinia-derived r-FIX (V/VrF-IX) and CHO-derived r-FIX (CHOOrF-IX), were obtained as pure proteins. The identity of the proteins was confirmed by N-terminal amino acid sequence analysis. Determination of the apparent molecular weights resulted in molecular weights of 72,000 Da (V/VrF-II) and 74,000 Da (CHOOrF-II and phF-II). CHOOrF-IX and V/VrF-IX exhibited identical apparent molecular weights of about 65,000 Da.

N-glycosylation of recombinant coagulation factors was confirmed by incubation of the proteins with N-glycosidase F to cleave asparagine-bound N-glycans. Deglycosylation resulted in the reduction of the molecular weights of both the plasma-derived and the recombinant coagulation factors, and resulting apparent molecular weights of about 68,000 Da and 60,000 Da for both recombinant and plasma-derived coagulation factors II and IX (Figure 3), respectively.

For detection of the N-glycosylation pattern, coagulation factor activity was determined by SDS-PAGE, electrobotted onto nitrocellulose, and then incubated with digoxigenin-labeled lectins. Lectins from *Datura stramonium* (DSA), *Galanthus nivalis* (GNA), *Maackia amurensis* (MAA), and *Sambucus nigra* (SNA) were used to detect galactose-(1-4)-N-acetylgalcosaminites, mannosamine-α(1-3)/(1-6)/(1-2)-mannose structures, sialic acid-α(2-3)-galactose units, and sialic acid-α(2-6)-galactose structures, respectively. Digoxigenin-labeled lectins were identified with alkaline phosphatase-conjugated sheep anti-digoxigenin immunoglobulin. Table 1 summarizes the detection of carbohydrate moieties of plasma-derived and recombinant coagulation factors.

High resolution anion exchange chromatography of V/VrF-II resulted in the separation of two glycosylation microheterogeneities. The V/VrF-II fraction iso-