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Analysis of Erythropoietin Glycoform Produced by Recombinant CHO Cells Using the Lectin-Blotting Technique

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The glycosylation pattern of Erythropoietin (EPO), produced by recombinant CHO cells, was studied using the simple and rapid technique of 'Lectin-blotting'. In this experiment we used three different kinds of lectins, MAA (*Maackia amurensis* agglutinine), RCA (*Ricinus communis* agglutinine), and DSA (*Datura stramonium* agglutinine), which bind to the terminal sialic acid, galactose, and the N-acetyllactosamine chain respectively. The lectin-blotting technique was used to analyze the carbohydrate structure of EPO produced in the presence of two physiologically active chemical compounds, ammonium and chloroquine. The effect of the ammonium ion on the glycosylation of EPO was studied because it accumulated in the medium mainly as a by-product of glutamine metabolism. Ammonium chloride significantly inhibited the sialylation of the terminal galactose residue at concentrations of 8 mM or more. Chloroquine, a potent inhibitor of glycosylation, inhibited terminal sialylation at concentrations of 100 and 200 μM, and at a concentration of 300 μM also inhibited N-acetyllactosamine chain synthesis.

Key words: Erythropoietin, CHO, carbohydrate, glycoform, lectin

INTRODUCTION

The structural integrity of oligosaccharides has attracted the attention of many industrial scientists and engineers because of recent recognition that environmental factors can affect the glycosylation of proteins in cell culture. Commonly encountered factors are glucose starvation, serum concentration and changes in extracellular pH [1, 2]. Ammonium ion, a metabolic by-product which accumulates in culture medium, has also been reported to disrupt oligosaccharide processing [3]. Growing interest in factors that may affect glycosylation makes it necessary for manufacturers to monitor glycosylation as a facet of quality control during the production of recombinant glycoprotein, in order to ensure the structural integrity of oligosaccharides. Therefore, glycosylation analysis techniques have become important in the structural characterization of glycoprotein.

EPO contains four long branched oligosaccharides that make up over 40% of molecular weight [4-6]. It was previously reported that the structure of the three N-linked oligosaccharides, which constituted the major portion of EPO carbohydrate, was closely related to its biological activity [7-10].

In this experiment, we characterized the structually important carbohydrate residues of Erythropoietin (EPO) using three different lectins, and examined the effect of two physiologically important chemical agents, chloroquine and ammonium chloride, on the glycosylation of EPO. Ammonium chloride was used to study the effect of the ammonium ion, since it accumulated in culture medium mainly as a by-product of glutamine metabolism. Chloroquine, a well-known glycosylation inhibitor, was used to confirm negative effects.

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MATERIALS AND METHODS

Cells and Their Maintenance

A culture of recombinant Chinese hamster ovary (CHO) cells expressing human EPO was kindly provided by Dr. H. J. Hong of the KRIBB (Korea Research Institute of Bioscience and Biotechnology). The cell line was constructed by introduction of genomic DNA encoding human EPO under the control of the Cytomegalovirus (CMV) promoter. Once the human EPO gene in the recombinant CHO cells had been amplified with dihydrofolate reductase (DHFR) by methotraxate selection, it was maintained in the medium without methotrexate. The cells were grown in Minimal Eagle's Medium alpha (MEMα) (Life technologies, NY, USA) supplemented with 2 g/L of D-glucose, and 10 % fetal bovine serum (FBS) (Gibco Laboratories, NY, USA). For the serum-free production medium, 10% fetal bovine serum was replaced with ITS (insulin 5 μg/mL, transferrin 5 μg/mL, sodium selenite 20 nM). Cells were cultured in a 75 cm^2 T-flask (Falcon, NJ, USA) and grown in a CO_2 incubator at 37°C with atmosphere of 5% CO_2 and 95% relative humid air.

Cell Culture under Treatment of Affective Agents

The culture was started with 2×10^6 cells in a T-75 flask with 20 mL of growth medium and incubated for 3 days, to allow it to become confluent, and to ensure that overgrowth of the cell monolayer did not occur. The medium was removed once the T-flasks were confluent and each flask was rinsed twice with phosphate buffered saline (0.2 g/L potassium phosphate monobasic, 0.2 g/L potassium chloride, 8.0 g/L sodium chlo-

ride and 1.15 g/L anhydrous sodium phosphate dibasic). The medium was then replaced with 20 mL of the serum-free production medium, supplemented with either 0, 4, 8, or 12 mM of ammonium chloride (NH_4Cl) and cultured for 12 hours. For the chloroquine treatment, 20 mL of the serum-free production medium supplemented with 0, 100, 200, or 300 μM of chloroquine was added, and cultured for 6 hours. Following the production phase, the medium was removed and stored in a freezer at − 20 °C. All experiments were duplicated.

SDS-PAGE and Lectin, Immunoblotting

Supernatant samples were concentrated 40-fold using 10,000 NMWL ultrafree-CL ultrafiltration membranes (Millipore, Bedford, MA, USA) then separated by SDS-PAGE (12% acrylamide gel) [11]. Once a sample had been separated, the protein bands were transferred to 0.45 micron nitocellulose membrane (Hybond C, Amersham, UK) in Transfer buffer (Tris 15.6 mM, Glycine 120 mM). After blocking the unoccupied protein binding sites on the nitrocellulose sheets with 1% bovine serum albumin (BSA) in TBS, the nitrocellulose sheets were incubated with the three types of digoxigenin (DIG) conjugated lectins (Table 1). The sheets were washed 3 times in TBST buffer and subsequently incubated with alkaline phosphatase conjugated anti-DIG antibody (Behringer Mannheim, Mannheim, Germany). Finally, the sheets were washed three times in TBST buffer and then incubated in 5-bromo-4-chloro-3-indol phosphate/4-nitroblue tetrazolium chloride (BCIP/NTP) reagent (Promega, WI, USA). To immunoblot the samples, DIG conjugated lectin and anti-DIG antibody and alkaline phosphatase conjugated anti-DIG antibody were replaced by monoclonal anti-EPO antibody and alkaline phosphatase conjugated anti-mouse IgG antibody (Promega, WI, USA), respectively.

RESULTS AND DISCUSSIONS

Production of EPO at Different Concentrations of Ammonium Chloride

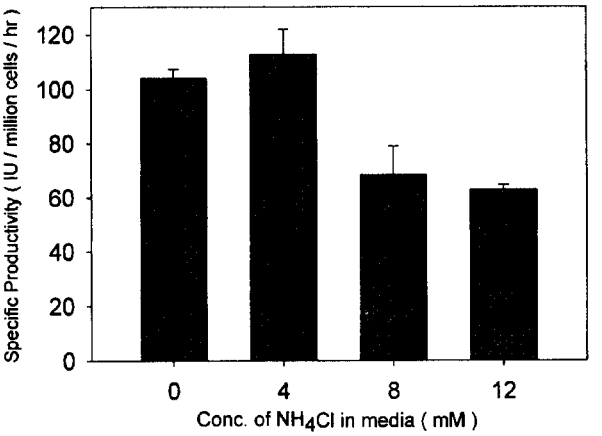

Fig. 1. Specific productibity of EPO in cultures containing various concentration of NH_4Cl.

The medium was removed after three days, when CHO cells became confluent, and the cells were rinsed twice with phosphate buffered saline. The cells were then cultured in a serum-free medium containing 0, 4, 8, or 12 mM of ammonium chloride to induce EPO production. After 12 hrs, the culture supernatant was harvested, and used to measure EPO titre, cell growth and viability, and specific EPO productivity. During culture, cell viability was maintained above 90% without a significant increase in cell numbers. The specific productivity of EPO was measured at 100-110 IU/10^6 cells/hr in the presence of ammonium chloride at concentrations up to 4 mM (Fig. 1). At higher concentrations, the specific productivity of EPO decreased significantly with ammonium chloride concentrations. Since cell viability was maintained above 90% throughout this experiment, it seemed that high concentrations of ammonium ion adversely affected the production of EPO.

The Interpretation of Blots

The pattern of the blot in different samples can provide information on N-glycan microheterogeneity (Fig. 2) [13, 14]. Since DSA (*Datura stramonium* aggluti-

Table 1. Lectins used in blotting experiment and their specificity

Lectin	Source	Specificity	Specificity on EPO
DSA	*Datura stramonium*	Galβ(1-4)GlcNAc GlcNAc-Ser / Thr	N-linked chain: N-acetyl-lactosamine chain O-linked chain: N-acetyl-glucosamine chain
MAA	*Maackia amurensis*	NeuNAcα(2-3)Gal	Terminal sialic acid residue
RCA	*Ricinus communis*	βGal > αGal	Galactose residue (rather than sialic acid) exposed at chain end (asialyo-chain)

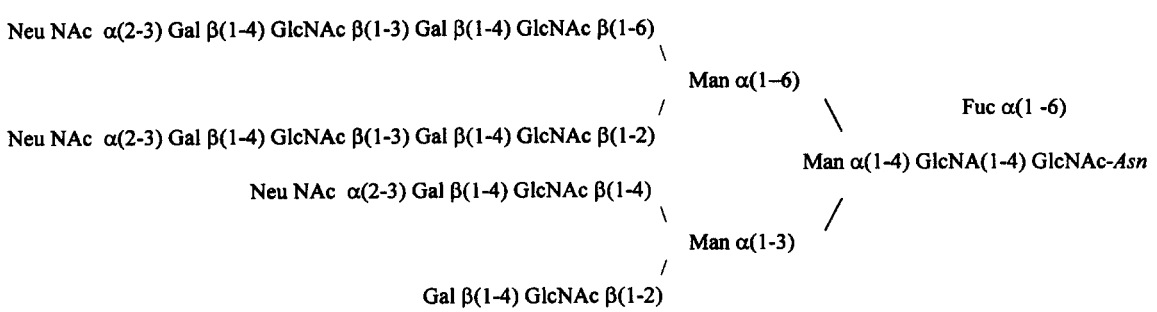

Fig. 2. Structure of major N-linked carbohydrate chains of EPO.

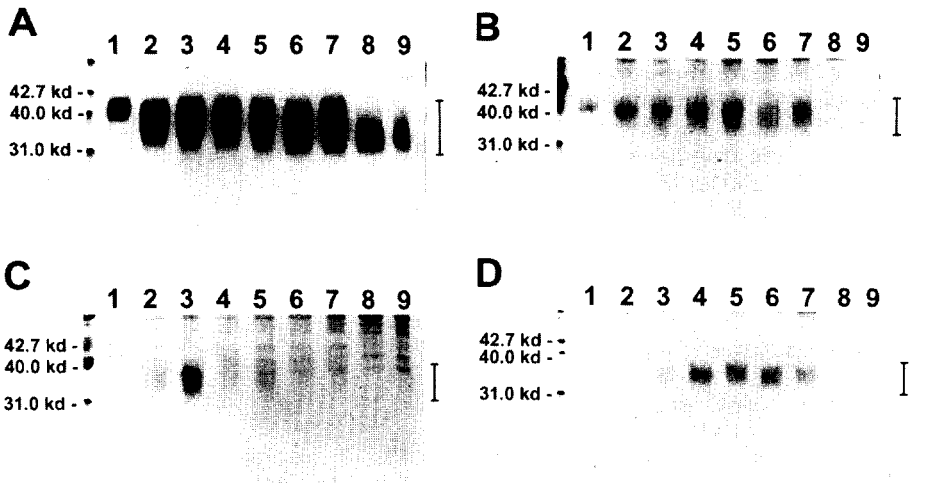

Fig. 3. Blot patterns of EPO produced in media supplemented with various concentration of NH_4Cl.(A) Immunoblots of anti-EPO antibody. (B) Lectin-blots of DSA. (C) Lectin-blots of MAA. (D) Lectin-blots of RCA. Lane 1: Authentic sample of EPO; lane 2, 3: culture sample without NH_4Cl; lane 4, 5: 4 mM NH_4Cl; lane 6, 7: 8 mM NH_4Cl; lane 8, 9: 12 mM NH_4Cl.

nine) binds primarily to N-acetyllactosamine chain (Galβ1-4GlcNAc), the structural integrity of N-acetyllactosamine residue in the sample can be verified with the blot. MAA (*Maackia amurensis* agglutinin) recognizes glycan when there is a sialic acid residue at the end of the glycan (NeuAcα2-3Gal) whereas RCA (*Ricius communis* agglutinine) recognizes a glycan end in which the sialic acid is missing, thereby exposing a galactose residue. Using these two lectins, MAA and RCA, in combination enables one to clearly determine the existence of a terminal sialic acid. It is important to evaluate the sialylation of EPO because there is a close relationship between the presence of sialic acid and the biological activity of EPO. Thus, it is possible to deduce structural information about the N-glycan of EPO by comparing the blot pattern of EPO produced in different conditions.

The supernatant of cultures supplemented with 0, 4, 8, or 12 mM of ammonium chloride was collected and analyzed by immunoblotting and lectin blotting. There were no significant changes in the immunoblots and

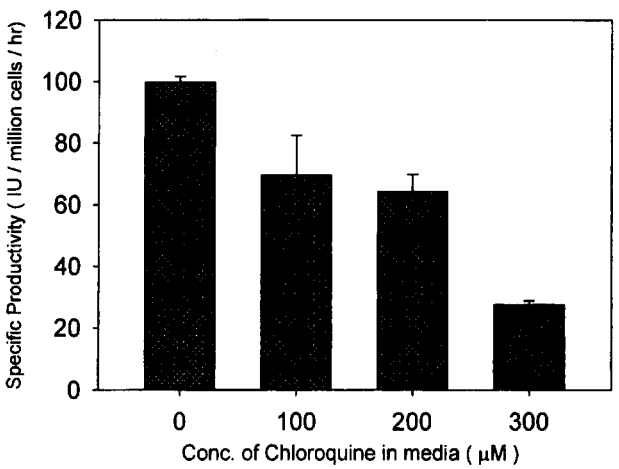

Fig. 4. Specific productivity of EPO in cultures containing various concentration of Chloroquine.

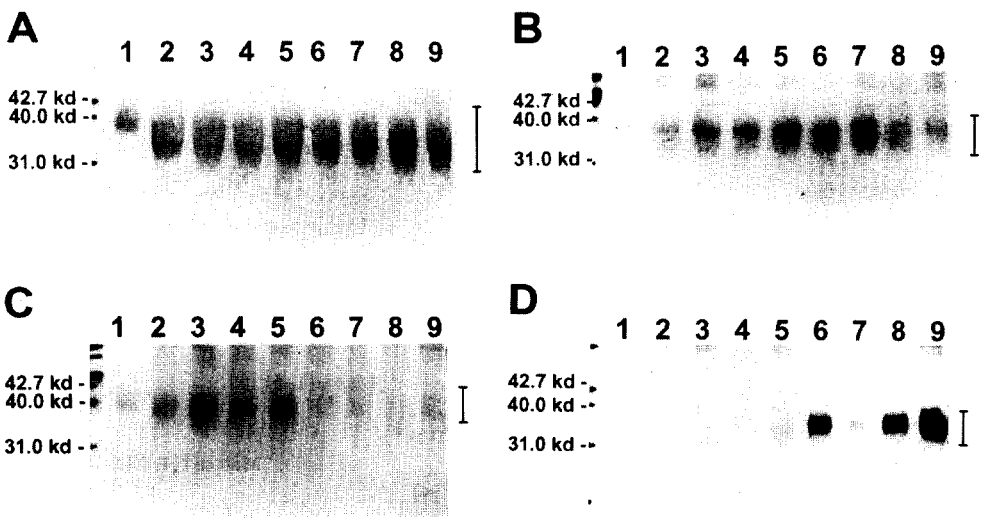

Fig. 5. Blot patterns of EPO produced in media supplemented with various concentration of chloroquine (A) Immunoblots of anti-EPO antibody. (B) Lectin-blots of DSA. (C) Lectin-blots of MAA. (D) Lectin-blots of RCA. Lane 1: Authentic sample of EPO; lane 2, 3: culture sample without chloroquine; lane 4, 5: 100 μM chloroquine; lane 6, 7: 200 μM chloroquine; lane 8, 9: 300 μM chloroquine.

the DSA lectin-blots over all concentrations of ammonium chloride (Figs. 3A and 3B). However, the results of lectin-blots using RCA and MAA were different. Labeling of the blot samples probed with MAA was notably less dense at concentrations of 8 and 12 mM, while the blots probed with RCA were clear at the same concentrations (Figs. 3C and 3D, lanes 6 to 9). These results indicate that ammonium chloride does not affect the synthesis of N-acetyllactosamine but strongly inhibits terminal sialylation at concentrations of greater than or equal to 8 mM. In perfusion culture systems, the ammonium ion concentration often becomes higher than 8 mM to affect terminal sialylation of EPO. It is therefore very important to look for glycoform change during the culture process.

Effect of Chloroquine on EPO Glycosylation

Cultures were incubated for 6hr in the presence of chloroquine at concentrations of 100, 200, and 300 μM using the same basic culture techniques described above for ammonium chloride. The specific productivity of EPO by CHO cells decreased inversely with the chloroquine concentration (Fig. 4). Cell viabilities of all cultures were maintained above 90% for the 6hr period, until the culture supernatant was replaced with serum-free medium.

The molecular weight of EPO decreased markedly at chloroquine levels of 300 μM (Fig. 5A, lanes 8, 9). This result suggests that a large portion of the glycoprotein was not fully synthesized, and it was assumed that the carbohydrate portion of EPO might be affected. The blots probed with DSA supported this hypothesis and showed that N-acetyllactosamine synthesis was inhibited at a concentration of 300 μM (Fig. 5B). Chloroquine concentration of higher than 300 μM completely inhibited terminal sialylation (Fig. 5C and 5D).

These experimental results demonstrate that lectin-blotting is a rapid and useful method for the qualitative evaluation of the glycosylation pattern of EPO and shows its potential for use in monitoring glycosylation of biological molecules.

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