Enhanced erythropoietin heterogeneity in a CHO culture is caused by proteolytic degradation and can be eliminated by a high glutamine level

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Received 4 August 1999; accepted 7 April 2000

Key words: CHO cells, erythropoietin, glycosylation, glutamine

Abstract
The molecular heterogeneity of recombinant human erythropoietin (EPO) increased during the course of a batch culture of transfected Chinese hamster ovary (CHO) cells grown in serum-free medium. This was shown by both an increased molecular weight and pI range of the isolated EPO at the end of the culture. However, analysis of the N-glycan structures of the molecule by fluorophore-assisted carbohydrate electrophoresis (FACE) and HPLC anion exchange chromatography indicated a consistent pattern of glycosylation. Seven glycoforms were identified, the predominant structure being a fully sialylated tetra-antennary glycan. The degree of sialylation was maintained throughout the culture. Analysis of the secreted EPO indicated a time-dependent increase in the molecular weight band width of the peptide consistent with proteolytic degradation. A high glutamine concentration (16–20 mM) in the culture decreased the apparent degradation of the EPO.

Introduction
Erythropoietin (EPO) is a 165 amino acid glycoprotein with two disulfide bonds and a molecular weight of 39 kDa (Jelkmann, 1992). Three N-linked and one O-linked oligosaccharide chains are attached to EPO polypeptide backbone and comprise 40% of the molecular weight of HuEPO (Sasaki et al., 1987).

The function of EPO is to stimulate the proliferation and differentiation of erythroid progenitor cells (Goldwasser and Kung, 1968). EPO is produced by the kidneys and is targeted to bone marrow (Jacobson et al., 1957). EPO affects early red progenitor cells through specific binding to one or more receptor molecules (Eridani, 1990). Recombinant EPO has been used for treatment of anemia associated with chronic renal failure, as well as some non-renal anemias. Treatment with EPO can restore normal hematocrit, eliminate the need for transfusions and improve the quality of life of its recipients (Ridley et al., 1994).

Large quantities of human EPO are required to satisfy the clinical demand for therapeutic use. Since primary cells produce only low concentrations of EPO, the human gene has been isolated, expressed and amplified in various mammalian cell lines (Jacobs et al., 1985; Lin et al., 1985). These transfected cell lines can form the basis of a production process. Product quality is extremely important for a molecule to be used therapeutically and any heterogeneity of a recombinant protein can be a serious problem.

Analysis of the effects of selective glycan removal from EPO has shown that the carbohydrate groups are essential for bioactivity in vivo (Dordal et al., 1985; Dubé et al., 1988; Narhi et al., 1991; Takeuchi et al., 1990; Tsuda et al., 1990). Of particular significance is the finding that the lack of terminal sialic acid (Neu5Ac) on the oligosaccharide structure results in high activity by in vitro bio-assays but low activity in vivo (Delorme et al., 1992; Narhi et al., 1991). This is explained by the presence of membrane receptors in the liver that can remove asialo-EPO from the circulatory system.

An important criterion for the development of a suitable production processes is to ensure the synthesis of a biologically active product that shows a consistent profile of heterogeneity. The heterogen-
eity of cell-secreted recombinant glycoproteins arises from two sources – variable peptide size and variable oligosaccharide structures. The heterogeneity of oligosaccharide structures can arise from variable intracellular processing as a result of various culture parameters, only some of which have been characterised. The culture parameters that may affect the variability of glycosylation include glucose depletion (Gershman and Robbins, 1981; Rearick et al., 1981), ammonia accumulation (Borys et al., 1994; Thorens and Vassalli, 1986), lipid composition (Castro et al., 1995; Jenkins et al., 1994), protein content (Castro et al., 1995) and pH (Borys et al., 1993).

Further heterogeneity is caused by proteases and glycosidases released from cells into the culture medium. These enzymes may degrade peptides and oligosaccharides in the culture medium. Thus a prolonged residence time of a secreted glycoprotein may result in some degradation which gives rise to further molecular heterogeneity (Gramer and Goochee, 1993; Gramer et al., 1995; Lind et al., 1991). It has been shown that the level of glycosylation can decrease over time in a batch culture as the chemical environment of the cell changes (Curling et al., 1990). Proteolytic degradation of the N-terminus of interferon-γ was shown to occur during its production in culture and this reduced its anti-viral activity (Hogrefe et al., 1989; Ichimori et al., 1987).

Serum contains specific protease inhibitors which can reduce product degradation in serum-supplemented cultures. However, the disadvantages in using serum in production processes, have led to the widespread use of low protein serum-free formulations for the growth of producer cell lines such as those transfected for synthesis of specific glycoproteins. In these cultures a reduction in proteolytic activity during cultivation of the cells may be achieved by addition of a protease inhibitor. However, these inhibitors are expensive and cause difficulty in product recovery (Schlaeger et al., 1987; Teige et al., 1994).

Other culture parameters may affect protease activity in the medium. It was reported that amino acid starvation (glutamine, asparagine, aspartate and serine) induced protease activity (Cartwright, 1994).

In the present study the heterogeneity of human EPO is determined during the batch culture of a transfected CHO cell line. The effect of glutamine concentration on this heterogeneity is also determined. Glutamine is an essential amino acid, a major energy source in proliferating mammalian cells and it serves as both a carbon and the nitrogen source (McKeehan, 1982; Reitzer et al., 1979). Glutamine in culture medium can prevent hybridoma and murine plasmacytoma cell apoptosis (Singh et al., 1994). Moreover, glutamine is involved in the biosynthesis of purines, pyrimidines and amino sugars, and the efficiency of recombinant protein glycosylation (Castro et al., 1995; Nyberg et al., 1999).

Materials and methods

Cell line and cultures

A stable CHO-K1 cell clone (EPO-81) transfected with the human EPO gene was provided by Cangene Corp. for this work. The cells were maintained in culture flasks in a humidified incubator at 37 °C and 10% carbon dioxide. Cell growth was in the serum-free medium designated CHO-SFM2.1. Cells were inoculated at 1 × 10^5 cells ml^-1 into 7 ml medium containing different concentrations of glutamine in 25 cm^2 T-flask. Cells were detached from the growth surface by trypsin (0.05%/3 min.). Viable cell concentration was determined by haemocytometer counting from each culture sample to which an equal volume of trypan blue (0.2%) was added. Culture supernatants were harvested and stored in −20 °C until analysis.

For EPO purification, cells were inoculated at 1 × 10^5 cells ml^-1 into 100 ml medium in 150 cm^2 T-flask. After 4 or 7 days culture, supernatants were collected and stored frozen.

Determination of culture concentrations of glucose, lactate, glutamine and ammonia

The glucose was determined by a kit from Sigma (Glucose Trinder 315-100). Glutamine was measured by a specific glutaminase assay based on a method previously described (Lund, 1985). Lactate was determined by a spectrophotometric assay using lactate dehydrogenase (Gutmann and Wahlefeld, 1974). Ammonia was measured by a gas-sensing electrode (Orion, model 95-12).

EPO determination by ELISA

EPO concentration in culture supernatant was determined by a sandwich ELISA as described previously (Yang and Butler, 2000). Briefly, polyclonal anti-human EPO (Sigma) was coated onto microtiter plates and then incubated with serial dilutions of EPO standard or culture supernatant samples. EPO was detected