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The availability of glucose to CHO cells affects the intracellular lipid-linked oligosaccharide distribution, site occupancy and the N-glycosylation profile of a monoclonal antibody

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ABSTRACT

The glycosylation pattern of a chimeric heavy chain antibody (EG2) produced from CHO cells was affected by the glucose concentration (0–25 mM) of cultures established at high density (>10^6 ml^{-1}) over 24 h. The resulting proportion of non-glycosylated Mab was directly correlated to the exposure time of cells to media depleted of glucose. Deprivation of glucose for the full 24 h resulted in a 45% non-glycosylated Mab fraction.

Analysis of steady state levels of intracellular lipid-linked oligosaccharides (LLOs) showed that under glucose limitation there was a reduction in the amount of full length LLO ($Glc_3Man_9GlcNac_2$), with a concomitant increase in the smaller mannosyl-glycans ($Man_{2-5}GlcNAc_2$). Glycan microheterogeneity was quantified by galactosylation and sialylation indices (GI and SI) which showed a direct correlation to the cell specific glucose uptake. The GI increased to 0.83 following media supplementation with a cocktail of uridine, manganese and galactose. This is significantly higher than for a fully humanized antibody (DP12) produced under the similar conditions or for similar antibodies reported in the literature. The high GI of the chimeric antibody (EG2) may be due to its low molecular weight and unusual structure. These findings are important in relation to the low substrate that may occur in fed-batch cultures for Mab production.

1. Introduction

The increasing demand for recombinant therapeutic proteins, especially recombinant monoclonal antibodies (Mabs), has been met by significant increases in productivity of mammalian cell culture bioprocess over the last decade (Butler, 2005; Wurm, 2004). Fed-batch culture has become the predominant strategy used for mammalian cell culture bioprocesses for biopharmaceutical production (Rouiller et al., 2013; Xie et al., 1997). The value of this strategy is to prolong the stationary phase of the culture in which viable cells are held at a high density in a productive state for several days.

Optimally designed feeding regimes with low concentrations of glucose and glutamine have been shown to lead to an efficient energy metabolism with minimal production of lactate and ammonia and a substantial increase of the productivity of a targeted protein (Dean and Reddy, 2013; Kim do et al., 2013). However, the precise feeding regime can lead to intermittent periods in which the substrates (notably glucose) may be reduced to levels that are critical to glycosylation (Xie et al., 1997).

N-glycosylation is the predominant type of post-translational modification that can affect the efficacy of a biopharmaceutical. Marked effects of the glycosylation profile of the glycoprotein include the enhancement of effector function (Jefferis, 2012), pharmacokinetics (Bumbaca et al., 2012), pharmacodistribution (Werner et al., 2007), antigenicity (Zhu et al., 2002), stability (Zheng et al., 2011) and solubility (Lee and Forciniti, 2013). Given the variety of potential effects of N-glycosylation on bioactivity of a glycoprotein, an understanding of the glycosylation machinery and the metabolic parameters that affect the heterogeneity of N-glycosylation during Mabs production is important to ensure the production of both a consistent glycosylation profile and one with a pre-determined clinical outcome.

N-glycosylation variations in mammalian cell bioprocesses may be found in the site-occupancy (macroheterogeneity) or glycan profile (microheterogeneity) as a result of various cell culture factors including nutrient limitation (Andersen et al., 2000; Curling et al., 1990). Structural variants of attached glycan structures produced

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from any cell are governed by a network of complementary and competing enzymes that do not always allow individual reactions to go to completion. This network is affected by multiple factors including the availability of precursor, co-factors and enzyme activities levels (Butler, 2006) all of which give rise to variable final glycan structure (microheterogeneity). The functional importance of the glycan profile has been recognized. Changes in glycan microheterogeneity in monoclonal antibodies, Mabs, have been recorded in vivo under physiological or pathological conditions. The enhancement of complement activation has been correlated with an increase in galactosylation of antibodies (Tsuchiya et al., 1989) and an enhanced anti-inflammatory effect with increased sialylation of antibodies (Kaneko et al., 2006; Scallon et al., 2007).

During Mab production from mammalian cell culture numerous parameters may have an impact on the heterogeneity of glycan structures. These include the availability of medium components, and culture environmental parameters. It has been known for some time that lower levels of glucose can lead to decreased site occupancy (Stark and Heath, 1979) or aberrant structure (Rearick et al., 1981). In one report of a fed-batch culture glucose <4 mM was shown to lead to extended periods of high cell viability through minimal by-product formation but further reduction (glc < 0.7 mM) resulted in decreased sialylation and the appearance of aberrant glycan structures of secreted gamma-interferon (Chee Furng Wong et al., 2005). Furthermore, microheterogeneity of glycans may be affected by various culture parameters (Butler, 2006) including the mode of culture (Andersen and Goochee, 1994), culture pH (Borys et al., 1993), specific media supplements (Crowell et al., 2008) or by-product accumulation (Gawlitzek et al., 2000). Typically, these variables affect the biosynthetic machinery within cells causing changes in the activity of glycosylating enzymes or the availability of critical metabolic precursors (Chen and Harcum, 2007).

Glucose and glutamine are precursors for cellular energy metabolism as well as for the synthesis of intracellular nucleotide-sugars that are essential substrates in N-glycosylation pathway. Glucose starvation has been shown to alter the biosynthesis of lipid-linked oligosaccharide (LLO) resulting in truncated precursors that may be transferred to proteins (Rearick et al., 1981a). This can lead to reduced site occupancy even in feeding regimes where there may be a temporary depletion in substrate concentrations (Hayter et al., 1992; Xie et al., 1997). The addition of specific substrates or co-factors has been shown to be a possible strategy to maximize terminal galactosylation (Grainger and James, 2013; Gramer et al., 2011) or sialylation (Gu and Wang, 1998).

In this report we investigated the effect of varying periods of glucose starvation on the profile of intracellular LLO and the final glycan profile of a chimeric Mab produced from Chinese hamster ovary (CHO) cells.

2. Materials and methods

2.1. Cell line

A stably transfected CHO (DUXB) cell line (CHO-EG2) expressing a chimeric human-llama heavy chain monoclonal antibody (cHCAb) against epidermal growth factor receptor (EGFR) was gratefully received from Yves Durocher, NRC, Montreal (Zhang et al., 2009). The antibody (designated EG2) is a fusion of a camelid single domain antibody to a humanized Fc fragment and has a molecular weight ~80 kDa. A second CHO cell line (DP12; clone #1934) was obtained from the American type culture collection (ATCC) for comparison of glycan profiles. This produces a human IgG Mab against interleukin 8.

2.2. Cell culture

The cells were maintained in cultures (80 mL) in 250 mL shake flasks in Biogro CHO media (Biogro Technologies Inc, Winnipeg, Canada) with 0.5 g/L yeast extract (BD, Sparks, USA) containing 25 mM glucose. The shake flasks were held in a humidified incubator at 120 rpm, 10% CO_2 and 37 °C.

A series of cultures were established at high density and variable glucose to determine differences in antibody glycosylation over 24 h. A pool of cells (2×10^8) was harvested following 3 days growth in standard culture media containing 25 mM glucose. These cells were inoculated at 2.6×10^6 cells/ml into 7 shake flasks (250 ml) each containing 80 ml of media with a different initial glucose concentration varying from 0 to 25 mM. A total of three trials were performed, with duplicate flasks at each glucose concentration. The high cell density at inoculation in these cultures was intended to ensure a rapid depletion of glucose from the media. The cultures were maintained and monitored under standard shaking conditions in an incubator over a 24 h period.

2.3. Cell culture sampling

Total cell counts and cell viability were measured at regular intervals by Trypan dye exclusion (0.5%) using a Cedex Image Analyzer (Innovates AG, Bielefeld, Germany). Glucose and lactate concentrations were determined enzymically using a YSI 2700 MBS Bioanalytical System (YSI Life Sciences, Yellow Springs, USA). The UMG supplements used to enhance galactosylation consisted of uridine (4 mM), manganese chloride ($MnCl_2$, 8 μM) and galactose (20 mM).

2.4. Antibody determination by ELISA

The EG2 antibody concentration was quantified by enzyme linked immunosorbent assay (ELISA). Goat produced anti-human IgG (Fc specific) antibodies (Sigma–Aldrich, St. Louis, USA, I21136) were used for coating ELISA plates (Invitrogen, Carlsbad, USA) in phosphate buffered saline (PBS) (Invitrogen, Carlsbad, USA) and blocked with PBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, USA) and 0.04% (w/v) Tween 20 (Sigma–Aldrich, St. Louis, USA, P1379). Supernatants of the culture samples were diluted with dilution buffer containing PBS, 0.5% (w/v) BSA and 0.04% Tween 20 and loaded onto the plates. An anti-human IgG (Fc specific) peroxidase-conjugated antibody produced in goat (Sigma–Aldrich, St. Louis, USA, A0170) was used for detection of EG2 antibody. The detection reagent consisted of 3,3′,5,5′-tetramethylbenzidine dihydrochloride tablet (Sigma–Aldrich, St. Louis, USA, T3405) and 4 μl of 30% hydrogen peroxide in 20 mL phosphate-citrate buffer (Sigma–Aldrich, St. Louis, USA, P4809). Sulphuric acid (2 M) was used as a stop solution. The titer was measured using an ELISA reader (Biotek, Winooski, USA) EL808 at 450 nm and Magellan software according to the instruction manual. The colorimetric measurements were converted to concentrations using a standard antibody solution (EG2) that was quantified by a Bradford assay and confirmed by a Lowry protein assay and ultraviolet absorption (A280).

2.5. Lipid-linked oligosaccharide (LLO) analysis

Cells were harvested and quenched to stop any subsequent metabolic activities (Sellick et al., 2009). LLOs were extracted from the cells using a previously established method (Gao and Lehrman, 2006). The chloroform/methanol/water fraction containing LLOs was subjected to mild acid hydrolysis and the released oligosaccharides were labeled with 2-aminobenzamide (2-AB; Sigma–Aldrich, St. Louis, USA) according to a previously developed method (Bigge