Changes in the overall extent of protein glycosylation by Chinese hamster ovary cells over the course of batch culture

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Glycosylation of recombinant proteins can change during the culture of animal cells. Since lipid-linked oligosaccharides (LLOs) are the carbohydrate donors in N-linked glycosylation, their availability is postulated to influence the extent of glycosylation. To test this hypothesis, relative LLO and glycosylation levels in Chinese hamster ovary (CHO) cells were monitored over the course of batch culture for corresponding changes. Radiolabelling studies reveal that throughout the length of culture, intracellular LLO levels remained within a 2-fold range and overall CHO protein glycosylation varied by less than 30%. The implications of these results and hypotheses to explain the findings are discussed.

Introduction

N-linked glycosylation is a post-translational modification commonly performed on proteins by eukaryotic cells [1] and it can significantly alter the efficacy of a human therapeutic protein [2]. The use of mammalian cells, such as Chinese hamster ovary (CHO) cells, for the commercial production of recombinant human proteins is often attributed to their ability to impart desired glycosylation features on proteins.

An essential step in N-linked glycosylation is the transfer of oligosaccharide from a lipid in the endoplasmic reticulum membrane to an asparagine residue within a specific amino acid consensus sequence on a nascent polypeptide [1]. In cultured cells, this reaction does not occur at every identical potential glycosylation site on different molecules of the same protein [3]. The resulting variation in the extent of glycosylation for a given protein is known as site occupancy heterogeneity.

Although current regulatory practice permits product heterogeneity, demonstration of specific and reproducible glycosylation is required [4]. Hence heterogeneity in protein glycosylation presents special challenges to the development and production of a candidate therapeutic with consistent properties.

In view of the inevitable occurrence and significance of glycosylation heterogeneity in protein therapeutics derived from mammalian cells, much research has been directed towards understanding factors that influence glycosylation heterogeneity during a bioprocess [2]. In particular, N-linked glycosylation site occupancy of human interferon-γ (IFN-γ) secreted by recombinant CHO cells has been the focus of many studies. Researchers from this laboratory and elsewhere have observed a gradual decline in IFN-γ glycosylation site occupancy over the course of batch and fed-batch cultures of recombinant CHO cells: the proportion of fully glycosylated IFN-γ decreased by 9–25% during the exponential growth phase [5–9]. This deterioration in glycosylation did not arise from extracellular degradation of product [6], nor could it be overcome by supplementation of the cultures with extra nutrients, such as nucleotide sugars [9], glucose and glutamine [6,10]. Certain lipid supplements minimized the glycosylation changes, but the underlying mode of action was not understood [11].

This work was conducted to investigate the intracellular changes responsible for the decline in IFN-γ N-linked glycosylation site occupancy over the course of CHO batch culture. Since lipid-linked oligosaccharides (LLOs) are the oligosaccharide donors in N-linked glycosylation, their availability may be a key regulatory mechanism for controlling the extent of protein glycosylation. Inadequate formation or excessive degradation of LLOs can result in LLO shortages and consequently limit cellular glycosylation capacity [12]. Under subsaturating LLO levels, a gradual decrease in the intracellular pool of LLOs would lead to a corresponding decrease in protein glycosylation. This scenario would account for the decline in IFN-γ glycosylation observed in CHO cells. To test this hypothesis, relative LLO and glycosylation levels in CHO cells were monitored over the course of batch culture to determine whether they exhibit corresponding changes.

Key words: G0/G1 phase, lipid-linked oligosaccharide.

Abbreviations used: CHO, Chinese hamster ovary; CMW, chloroform/methanol/water (10:10:3, by vol.); IFN-γ, interferon-γ; LLO, lipid-linked oligosaccharide; TCA, trichloroacetic acid; tPA, tissue plasminogen activator.

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Materials and methods

CHO cell line

The recombinant CHO cell line used in this work has been described previously [13,14]. This dihydrofolate reductase-deficient CHO cell line was created by co-transfection with genes for dihydrofolate reductase and human IFN-γ [15]. It was subsequently adapted for anchorage-independent growth in two different serum-free media, either CHO-S-SFM II (Gibco BRL) supplemented with 0.25 μM methotrexate, 10,000 units/l penicillin and 10 mg/l streptomycin [13] or RPMI SFM [14], a medium developed in this laboratory based on RPMI-1640 (Sigma). The cells were typically cultured in shake flasks agitated at 70 rev./min on orbital shakers in a 37 °C incubator with 95% humidity and 5–10% CO₂ overlay.

Radiolabelled CHO batch cultures

In preparation for the first radiolabelling study, a vial of frozen CHO cells adapted to grow in CHO-S-SFM II was taken from a working cell bank. The cells were inoculated at 3 × 10⁶ cells/ml and subsequently passaged thrice. The cultures were consistently maintained at densities of (3–10) × 10⁶ cells/ml with viabilities exceeding 95%. Radiolabelling medium was prepared by supplementing the CHO-S-SFM II basal medium with 6.7 μCi/ml d-[U-14C]glucose (American Radiolabelled Chemicals) and 2.6 μCi/ml L-[4,5-3H]leucine (Amersham Biosciences).

Prior to starting this time-course study, the cells were resuspended in the radiolabelling medium. After 26 h of incubation, the conditioned radiolabelled medium was separated from the cells by centrifugation (5 min, 200 g) and discarded. To start the time-course study, the prelabelled CHO cells were first resuspended in sufficient fresh labelling medium to yield a cell density of 3 × 10⁵ cells/ml and then divided into duplicate shake flasks.

At 12 h intervals throughout the experiment samples containing (5–10) × 10⁶ cells were taken from the parallel batch cultures for various measurements. Cells were first sedimented by centrifugation (5 min, 200 g), and then used for cell enumeration, for cell-cycle analyses and for the extraction of LLOs and cellular proteins. The supernatant was filtered and stored at −20 °C. Subsequently, the supernatant was thawed for glucose measurements and for the extraction of secreted proteins. The experiment was terminated when culture viabilities dropped below 60%.

This experiment was repeated with two modifications in a second radiolabelling study. First, the radiolabels used were d-[2-3H]mannose (American Radiolabelled Chemicals) and l-[5,5S]methionine (Amersham Biosciences), such that their final concentrations in the CHO-S-SFM II medium were 97 and 0.9 μCi/ml, respectively. Second, in contrast to the previous experiment, the CHO cells were not prelabelled prior to starting this time-course study.

Cell and metabolite analyses

Cell densities were determined by using a haemacytometer to count cells. Trypan Blue dye exclusion was used for the determination of culture viabilities. To minimize random errors, at least 500 cells were counted for each culture sample. Cell-cycle distributions were analysed by flow cytometry of cells stained with propidium iodide [16]. Glucose concentrations in filtered cell culture supernatants were measured directly by the YSI 23000 STAT Plus Glucose & Lactate Analyser (YSI Life Sciences).

Isolation of LLOs and cellular proteins

LLOs and cellular proteins were isolated following a method described previously [17]. In particular, LLOs were isolated essentially as described previously for CHO cells [18]. This sequential extraction procedure is based on the fact that LLO is insoluble in chloroform/methanol (2:1, v/v) and water, but soluble in chloroform/methanol/water (10:10:3, v/v; CMW) [19,20]. Briefly, each cell pellet was first washed three times with 6 ml of chloroform/methanol (2:1, v/v). The delipidated cell pellet was then rinsed thrice with 7 ml of water to remove water-soluble radioactivity. Finally, LLOs were extracted from the cell pellet with three washes of 7 ml of CMW and transferred into a glass scintillation vial. After the extraction solvent was evaporated to dryness in a fume hood, and 20 ml of Ultima Gold LSC cocktail (Packard Bioscience) was added to each scintillation vial to dissolve the LLOs in preparation for liquid scintillation counting.

The residue remaining after the lipid-extraction process contained cellular proteins [21]. After this pellet was air dried, it was dispersed in 1 ml of 10% (w/v) trichloroacetic acid (TCA) by sonication. Ice-cold 10% (w/v) TCA (7 ml) was added and the mixture was allowed to stand for a few minutes at 4 °C. Protein precipitated by the acid was sedimented by centrifugation (4 min, 1200 g). The pellet was subsequently washed with 10 ml of ice-cold 10% (w/v) TCA and 10 ml of ice-cold water. The final protein precipitate was first digested by warming with 0.5 ml of 1 M NaOH in a 50 °C water bath to give a clear colorless solution before being dissolved in 20 ml of Ultima Gold LSC cocktail and transferred to a scintillation vial for radioactivity measurements.

When the isolation procedure used in this work was performed on mammalian systems, the CMW extracts and delipidated residue were assumed to consist primarily of LLOs [17,22–24] and proteins [17,20,21,25], respectively.
Extraction of secreted proteins

Samples of filtered cell culture supernatants (3–5 ml) that had been frozen at −20 °C were thawed at room temperature. An equal volume of ice-cold 20% (w/v) TCA was added to each sample to precipitate the protein. The solutions were mixed and allowed to sit at 4 °C for 1 h prior to centrifugation (4 °C, 5 min, 1200 g). The TCA-soluble wash was discarded and the TCA-precipitable material was rinsed twice with 8 ml of ice-cold 10% (w/v) TCA. To prepare the final residue for liquid scintillation counting, it was first solubilized in 0.5 ml of warm 1 M NaOH and then mixed with 20 ml of Ultima Gold LSC cocktail in a scintillation vial. The incorporation of radiolabels into secreted proteins was determined as TCA-insoluble material in the culture medium [25,26].

Radioactivity measurements

Radioactivity was quantified using an LS 6500 Liquid Scintillation Counter (Beckman Instruments).

Results and discussion

Radiolabelling of LLOs and proteins

The procedure for LLO extraction from mammalian cells is well established [17–25]. However, the absolute amount of LLOs in CHO cells is difficult measure because it is very small: at least $4 \times 10^8$ CHO cells were required to supply enough LLOs (0.2–0.5 nmol) for quantification by direct sugar analysis [27]. Therefore, LLOs have typically been studied by radiolabelling of their sugar or lipid components [18].

Spiro and co-workers [28–30] conducted detailed studies on radioactive LLOs harvested from various mammalian systems using the sequential lipid-extraction procedure employed in this work. The LLOs were labelled by incubating the tissue slices with $^{14}$C-glucose. Paper chromatography of CMW extracts in numerous solvent systems revealed a single peak [28]. DEAE-cellulose chromatography of the CMW extracts obtained from several mammalian tissues demonstrated that the CMW fraction was essentially composed of LLOs [28–30]. For instance, a single $^{14}$C radioactive peak was observed when CMW extracts of tissue incubated in $^{14}$C-glucose was fractionated on a DEAE-cellulose column [28]. In particular, the LLO peak accounted for 86% of the $^{14}$C radioactivity from DEAE-cellulose chromatography of pancreas extract and 95% of that from the oviduct [30]. Incubations of the tissue slices with $^{14}$C-mannose or $^{14}$C-glucosamine yielded the same qualitative results [28].

Other researchers have further characterized the CMW fractions isolated by the sequential lipid-extraction method described in this work and have consistently found CMW extracts to contain primarily LLOs [22,31,32]. For instance, when Hubbard and Robbins [31] used $^{3}$H-mannose as the radiolabel, only a trace of the radioactivity measured in the washed CMW fraction was free $^{3}$H-mannose and the authors subsequently labelled the CMW extracts as LLOs after further analyses [31]. When Kabakoff and co-workers [33] used $^{14}$C-mannosylphosphoryldolichol to spike CHO cell pellets used for LLO extraction, no significant amount of $^{14}$C radioactivity was detected in CMW extracts [33]. However, significant $^{3}$H radioactivity was measured in the LLO extracted from these CHO cells that had been cultured in the presence of $[2-^{3}$H]mannose.

In assaying N-glycosylation activity in cultured B-cells by measuring the incorporation of $[2-^{3}$H]mannose into LLO and glycoproteins, Rush and co-workers [32] performed a detailed analysis of the CMW and residue. They identified the radioactivity extracted into CMW washes as LLO by chemical and chromatographic properties and verified that the $^{3}$H-labelled product recovered in the delipidated residue was glucoprotein using pronase digestions and gel chromatography. In addition, they found that the incorporation of $^{3}$H radioactivity into both the CMW and the delipidated residue fractions was inhibited by 90–100% by tunicamycin at 2 μg/ml. Waechter and co-workers [22] demonstrated that the CMW and delipidated residue isolated from neuroblasts cells were composed essentially of LLOs and proteins, respectively. In view of these conclusive findings, researchers have assumed that CMW extracts are comprised of LLOs [17,22–24] and that delipidated residues are composed of proteins [17,20,21,25].

In a thorough investigation into the incorporation of radioactivity into the various sugar constituents of both lipid and protein fractions, Spiro and co-workers [29] found that incubation of calf thyroid slices with $[^{14}]C$ monosaccharides yielded $^{14}$C-labelled LLOs. The neutral sugar components were labelled throughout the oligosaccharide portion of LLOs. In particular, when $[^{14}]C$-glucose or $[^{14}]C$-mannose was used as a substrate, a substantial amount of radioactivity was incorporated and the neutral sugar components became labelled throughout the oligosaccharide, such that when degradative procedures were applied for the purpose of characterization all of the fragments contained radioactivity. The labelling of individual monosaccharides on LLOs was found to be qualitatively quite similar whether $[^{14}]C$-glucose, $[^{14}]C$-galactose or $[^{14}]C$-mannose was used as the substrate, although the absolute conversions were different, presumably due to variations in pool sizes and activity of enzymes involved in the saccharide conversions. A substantial amount of $^{14}$C radioactivity was also incorporated into the sugar components on glycoproteins.

Incubations of calf thyroid slices with radioactive glucose showed that more than 3 h were required for both
the glucose and mannose residues of the LLO to be maximally labelled [29]. Studies in mice uterine tissues indicated that the incorporation of radiolabelled sugars into LLOs reached maximal levels only after 4 h of incubation [34]. These results suggest that the time required for LLO levels to be labelled to metabolic equilibrium with radioactive sugars is in the order of hours in mammalian cells. This length of time is consistent with the numerous saccharide interconversions and nucleotide sugar syntheses required to label the various sugar residues on LLOs.

In the first experiment, [14C]glucose and [3H]leucine were chosen as the radioactive supplements because their unlabelled forms are normal components of the cell culture medium. By keeping the amounts of radiolabelled nutrients at extremely low concentrations relative to the quantities of their unlabelled counterparts in the medium, possible perturbations to the usual culture process were minimized. The subsequent cellular uptake and metabolism of [14C]glucose and [3H]leucine would generate 14C-labelled LLOs and glycoproteins as well as 3H-labelled proteins. Assuming that there is no preferential uptake of [14C]glucose over unlabelled glucose by the cells, the supplemental radiolabelled glucose should not be exhausted before the end of culture since the concentration of glucose in the culture medium would remain consistently above 1.3 g/l (7.2 mM) throughout the batch process (results not shown). A similar assumption was made for the radiolabelled leucine.

CHO cells were pre-incubated in the labelling medium for 26 h in the first experiment. This incubation period should be sufficient to label both the intracellular LLOs and cellular proteins to a steady state in the CHO cells prior to the transfer of the actively growing cells into fresh labelling medium at the start of the time-course experiment. The 14C radioactivity in LLOs extracted from the cells throughout the length of the experiment provides an indication of the relative LLO levels by measuring the amount of radiolabelled sugars present. The ratio of 14C/3H radioactivity in protein samples denotes the abundance of sugars relative to amino acids and thereby represents the extent of protein glycosylation.

This time-course study was repeated using [2-3H]mannose and [35S]methionine as the radiolabelled supplements to validate the use of [14C]glucose and [3H]leucine in the first experiment as well as to substantiate the findings.

Cell density and viability
The duplicate batch cultures of CHO cells demonstrated corresponding declines in total cell densities and viabilities (Figure 1). Total cell densities peaked at 60 h, and rapidly declined thereafter. Culture viabilities started to decrease below 95% after 48 h of cultivation. The experiment was terminated when culture viabilities decreased to 50% at 96 h.

Cell-cycle distributions
The cell-cycle distributions of the CHO cells were measurable up to 72 h of cultivation (Figure 2). After 72 h in batch culture, over a quarter of the cells were dead (Figure 1). The resulting accumulation of cell debris prevented accurate determination of cell-cycle distributions in subsequent samples.

The first datum point used for cell-cycle analyses (taken at 0 h) was obtained from CHO cells that had been growing exponentially for 26 h. At the start of this experiment (0 h) these cells were transferred into fresh medium. Cell-cycle distributions measured at 0 h were predictably similar to those recorded at 24 and 36 h, since all three sets of data were generated from cells that had been growing in fresh medium for approx. 1 day. The exposure to fresh medium
Glycosylation in Chinese hamster ovary cell batch cultures

Figure 3 Relative LLO levels in CHO cells over time in duplicate batch cultures spiked with [14C]glucose

14C radioactivity measured in LLOs extracted from CHO cells at regular time intervals was normalized to total cell number (A), viable cell number (B) and [3H]leucine radioactivity in cellular proteins (C). 14C radioactivity originated from 14C-labelled supplemental glucose and was counted in disintegrations/min (DPM). Each data point was generated from identical parallel CHO batch cultures supplemented with [14C]glucose and [3H]leucine. Error bars indicate the differences in measurements between samples obtained from the duplicate cultures.

stimulated cell growth, as confirmed by the maximum percentage of S phase cells measured at 12 h. Subsequently, the percentage of G0/G1 phase cells gradually increased while the percentage of S phase cells declined.

Relative LLO levels over culture time

In the first experiment employing [14C]glucose and [1H]leucine, normalization of LLO measurements to total cell number, viable cell number or [3H]leucine radioactivity in cellular proteins generated similar results (Figure 3). The level of 14C radioactivity in LLO extracts per million cells gradually decreased by about 50% over the course of culture (Figure 3A). The ratio of 14C radioactivity in LLO extracts to the [1H]leucine radioactivity in the cellular protein extracts also showed a similar decline with culture time (Figure 3C). However, normalization of LLO 14C radioactivity to viable cell number showed that the relative LLO level per viable cells remained fairly constant over the duration of culture (Figure 3B). This suggests that the gradual decline in LLO levels relative to total cell numbers or cell protein resulted from the concomitant decline in cell viability.

When the time-course study was repeated using [2-3H]mannose and [35S]methionine, no decline in the level of 3H radioactivity in the LLO extracts was observed (Figure 4A). Together, these results show that CHO cells reproducibly maintained their LLO levels within a 2-fold range over the course of batch culture. However, the multi-step sequential LLO extraction procedure was not sensitive enough to provide reliable quantification of smaller variations in LLO levels since the measurements obtained had significant error margins. Hence it is possible that fractional changes in LLO levels did occur.

Studies have identified a cell-cycle dependence of LLO biosynthesis in rat 3Y1 cells [35] and human fibroblasts [36]. In these cells, LLO levels were at least 10 times higher during the S phase than during other phases of the cell cycle. Although the results here demonstrate conclusively that LLO abundance remained within an order of magnitude throughout the course of CHO cell culture, they do not convincingly argue for or against an association between LLO levels and the cell cycle.

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Relative glycosylation levels over culture time

When hybridoma cells were incubated in the presence of [2-3H]mannose and [35S]methionine, the cellular incorporation of TCA-precipitable radioactivities was measured and the ratio of 3H to 35S radioactivities was used to represent the glycoprotein biosynthesis rate [24]. A similar approach was taken in this work.

In the first experiment, supplemental [14C]glucose and [3H]leucine were used as the radiolabels. To determine the relative extent of protein glycosylation, incorporation of 14C and 3H radioactivities into TCA-insoluble material was assayed. The 14C to 3H ratios measured at each time point were always higher in secreted proteins than in cellular proteins (Figure 5), in accord with the higher likelihood for glycosylation. Upon the onset of massive cell death, 14C to 3H ratios in proteins began to descend from their maximum values, consistent with the expectation that dying cells have impaired glycosylation machinery. Towards the end of culture, the 14C to 3H ratios in secreted proteins approached that obtained for cellular proteins (Figure 5). This decline in glycosylation is attributed to the release of intracellular proteins by cell lyses. Since the relative glycosylation of secreted proteins was calculated by precipitating all the proteins accumulated in the culture medium, the addition of non-glycosylated intracellular proteins such as cytosolic and nuclear proteins to the pool of secreted proteins would lower the ratio of monosaccharides to amino acids measured in proteins extracted from the medium. When this experiment was repeated using [2-3H]mannose and [35S]methionine as the radiolabels, the same glycosylation trends were observed (Figure 4B).

Prior to the significant loss of cell viability in the CHO cultures, the ratio of labelled sugars to amino acids in both cellular and secreted proteins changed over the course of batch culture (Figures 4B and 5) in a similar pattern to that observed for the percentage of G0/G1-phase cells in the culture (Figure 2). Both sets of values initially decreased, but the subsequent increase in the proportion of G0/G1-phase cells was followed by a corresponding elevation in the 14C to 3H ratios for both cellular and secreted proteins.

The results here corroborate previously observed correlations between tissue plasminogen activator (tPA) glycosylation site occupancy and the fraction of cells in the G0/G1 phase of the cell cycle [37]. It is plausible that with the slowing down of cell growth (evidenced by the increase in the percentage of G0/G1-phase cells and the co-ordinate decrease in percentage of S-phase cells), net protein productivity declined. At the same time, if intracellular LLO levels remained constant, more LLOs would be available per protein for oligosaccharide transfer and thereby lead to an overall improvement in protein glycosylation.

The critical role of protein elongation rate in controlling glycosylation site occupancy presents another mechanism to explain the positive association between glycosylation efficiency and the proportion of G0/G1-phase cells in the culture [38]. This alternative hypothesis assumes a retardation in protein elongation rate during translation under conditions of reduced growth. The resulting increase in contact time between the protein and oligosaccharide substrate would enhance the success rate of the enzyme-catalysed glycosyl transfer reaction and thereby improve the extent of glycosylation.

Although the ratio of radiolabelled sugars to radiolabelled amino acids in both cellular and secreted proteins varied by less than 30% throughout the course of culture, these values showed overall increases over time (Figures 4B and 5). The error margins associated with the duplicate batch cultures were tight enough to support the validity of the observed glycosylation trends. Moreover, when this time-course experiment was repeated using CHO cells cultured in a different culture medium (RPMI SFM), qualitatively similar results were obtained: relative LLO levels remained within a 2-fold range and relative glycosylation levels displayed identical changes over time (results not shown). The possibility that LLOs and glycosylation levels were limited by glucose availability can be eliminated since the CHO-S-SFM II culture medium consistently contained at least 1.3 g/l (7.2 mM) glucose throughout the experiment (results not shown).

These results suggest that these CHO cells glycosylate their proteins to a gradually increasing extent as culture progresses, until the onset of massive cell death. The 15–25% improvement in overall protein glycosylation observed here is in agreement with that reported for CHO-derived
recombinant tPA [37]. Andersen and co-workers [37] observed an increase in the glycosylation site occupancy of tPA produced by CHO cells over the course of fed-batch culture. The pattern and extent of the tPA site occupancy enhancement were similar to that observed here with overall CHO protein glycosylation. However, these glycosylation trends contradict those observed specifically of IFN-γ produced by CHO batch and fed-batch cultures [5–9]. In particular, previous research conducted on the same CHO cell line used in this work showed that the percentage of fully glycosylated IFN-γ produced by CHO batch cultures declined by 15–25% over time [9]. To reconcile these divergent findings, we propose that the glycosylation site occupancy of different proteins may undergo distinct changes over the length of culture even though the net glycosylation efficiency in CHO cells improves with cultivation time. This proposition has strong implications for recombinant protein production using CHO cells: the glycosylation pattern of each individual glycoprotein product needs to be tracked over the course of culture because different proteins may exhibit different glycosylation variations with time, even when the same culture method is used.

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