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Visualisation of intracellular production bottlenecks in suspension-adapted CHO cells producing complex biopharmaceuticals using fluorescence microscopy

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ABSTRACT

With the advance of complex biological formats such as bispecific antibodies or fusion proteins, mammalian expression systems often show low performance. Described determining factors may be accumulation or haltering of heterologous proteins within the different cellular compartments disturbing transport or secretion. In case of the investigated bispecific antibody (bsAb)-producing Chinese hamster ovary (CHO) cell line neither impaired transcription nor decreased translation processes were identified and thus satisfactorily explained its low production capacity. Hence, we established a streamlined confocal microscopy-based methodology for CHO production cells investigating the distribution of the recombinant protein within the respective organelles of the secretory pathway and visualised the structure of the endoplasmic reticulum (ER) to be affected pinpointing towards an intra-ER bottleneck putatively hampering or limiting efficient secretion. The ER displayed not only a heavily altered morphology in comparison to a high immunoglobulin G (IgG)-producing cell line with a possibly inflated or overloaded structure, but the recombinant protein was also completely absent in the Golgi apparatus. Notably, the results obtained using an automated microscopy approach suggest the possible application of this methodology in cell line development and engineering.

1. Introduction

Monoclonal antibodies are generally well-tolerated biopharmaceuticals highly specific in activating, inhibiting or blocking molecular targets to modulate diseases. Even though the optimisation of production processes for therapeutic antibodies is a continuing challenge, cell line development approaches establishing high-yielding production cell systems have been thoroughly optimised during the past decades, resulting in markedly streamlined developmental process timelines (Kunert and Reinhart, 2016). The clinical and commercial success of monoclonal antibodies led to a continuous effort to generate innovative and promising non-naturally existing antibody-derived biological formats. Many molecules are designed to be bispecific or multivalent for example to retarget T cells to kill tumour cells or to interact with different disease mediators (Spiess et al., 2015). At least 30 different antibody-derived biological formats are currently in clinical development and even more intriguing formats are under investigations (Spiess et al., 2015). As the generation of stable mammalian production cells requires a considerable investment of time and resources, the respective antibody encoding genes are first transiently expressed to test its efficacy and manufacturability, especially in case of artificial antibody-derived biological formats (Kim et al., 2012). However, in case of these more complex biological formats, mammalian expression systems often show low performance with cell lines displaying inferior product titers, low specific productivity or insufficient product quality as they may have artificial domain-specific folding, processing and/or secretion requirements (Lee et al., 2007).

All canonical steps in protein production such as transcription, translation, post-translational modification and intracellular protein transport are tightly regulated in the wild-type host cell line and will

Abbreviations: bsAb, bispecific antibody; CHO, chinese hamster ovary; ER, endoplasmic reticulum; IgG, Immunoglobulin G; DTE, difficult-to-express; DHFR, dihydrofolate reductase; MTX, Methotrexate; VCD, viable cell density; qRT-PCR, quantitative real-time polymerase chain reaction; HC, heavy chain; LC, light chain; DAPI, 4′,6-Diamidin-2′-phenylindol; SD, standard deviation

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impact the specific productivity of any producer cell line derived from this host. Any rate limiting step during production might represent a bottleneck for the final secretion of proteins into the culture medium. Especially, during production of difficult-to-express (DTE) molecules, which may accumulate or be haltered within the different cellular compartments and transport or secretion may be disturbed (Le Fourn et al., 2014). Furthermore, non-optimised heterologous proteins might be degraded intracellularly by the ubiquitin proteasome system or the unfolded protein response machinery (Johari et al., 2015). Over the past decades, several cellular pathways and processes have been identified as putative rate-limiting steps in mammalian production cell lines resulting in low product quantities or quality. Investigated pathways and mechanisms include translation (Kallehauge et al., 2017), folding and assembly (Pybus et al., 2014, O'Callaghan et al., 2010), intracellular transport and secretion (Florin et al., 2009, Peng and Fussenegger, 2009; Peng et al., 2010, 2011) as well as aggregation (Hasegawa et al., 2011, 2017; Stoops et al., 2012) and degradation (Johari et al., 2015) of the produced recombinant proteins. Generation of energy as well as precursor molecules can also represent a bottleneck (Dickson, 2014; Sellick et al., 2011). However, when comparing different cell lines and products, at best similar pathways but not the same genes were identified to represent rate-limiting steps and thus bottlenecks may be very specific to a certain molecule or production cell line.

Consequently, there is an urgent need to identify rate-limiting steps and de-bottleneck current expression systems especially in the context of novel emerging protein formats and engineer superior cell factories with improved properties as a basis for efficient and high yielding manufacturing processes. Therefore we here provide a streamlined fluorescence microscopy based methodology optimised for CHO production cells tracing the recombinantly produced protein traveling through the distinct cellular compartments of the secretory pathway. Visualised possibly molecule or production cell specific rate-limiting steps in recombinant protein production can be used as a starting point for cell engineering. Investigated organelles include the nucleus, the ER and the Cis-Golgi as well as lysosomes and endosomes facilitating the identification of repressive or saturated organelle structures hampering efficient protein production. We further demonstrate that using an automated high-throughput fluorescence microscope enables screening of production rate limiting steps in CHO production cell lines.

2. Materials and methods

2.1. Cell line generation

For generation of the investigated bsAb-producing and IgG-producing cell lines Boehringer Ingelheim proprietary suspension-adapted CHO DG44 dihydrofolate reductase (DHFR)-deficient host cells were transfected with two different expression plasmids. Beside DHFR and aminoglycoside phosphotransferase expression cassettes, respectively, the expression plasmids code for a classical IgG1 antibody and the individual chains of a symmetric, bispecific antibody molecule using similar regulatory elements, respectively. Afterwards IgG1- and bsAb-positive cells were selected in hypoxanthine and thymidine-deficient medium supplemented with 200 mg/L Geneticin as well as 200 nM Methotrexate (MTX) and 100 nM MTX, respectively.

2.2. Cell culture maintenance

Suspension-adapted CHO DG44 cells were cultured in Boehringer Ingelheim proprietary serum-free, chemically-defined and animal component free media (Boehringer Ingelheim, Biberach, Germany). The cultivation was performed in shake flasks (Corning, Oneonta, NY, USA) with 120 rpm (50 mm orbit) in an orbital shaker incubator (Infors, Bottmingen, Switzerland) at 37 °C and a relative CO_2 concentration of 5 %. Cell cultures were seeded with a viable cell density (VCD) of 3×10^5 cells/ml and passaged every 2–3 days. Cell concentration was measured using the Cedex device (Roche Diagnostics, Indianapolis, IN, USA) by means of trypan blue exclusion.

2.3. Fed-batch cultivation

Cells were seeded with a VCD of 3×10^5 cells/ml in an initial volume of 250 ml in a 1000 ml shake flask (Corning) and incubated as mentioned before. For each liter of total culture volume 30 ml feed medium (Boehringer Ingelheim) were added daily. 72 h post-seeding the relative CO_2 concentration was shifted to 2 %. Product concentration was determined using the Octet® device by FortéBio® (Menlo Park, CA, USA). The Biosen S-line analyser (EKF Diagnostics, Cardiff, UK) was used for measurement of glucose and lactate concentrations. Glucose bolus was added (Boehringer Ingelheim) when the concentration fell below a pre-defined critical value of 3.0 g/l.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

The isolation of total RNA from cell cultures was robotically processed using the automated QIAsymphony® platform (Qiagen, Hilden, Germany). Afterwards, concentrations were determined with a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For reverse transcription, a final concentration of 2.5 ng/µl total RNA was used for each reaction performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Final qRT-PCR was done with the TaqMan® Gene Expression Master Mix (Applied Biosystems) processed on a Rotor-Gene® Q (Qiagen). TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems) were used to detect 18S ribosomal RNA as an endogenous control.

2.5. Western blotting

For protein analyses cells were lysed by incubation for 25 min at 23 °C in Passive Lysis Buffer (Promega, Madison, WI, USA) agitated at 600 rpm in an orbital shaker (Eppendorf, Hamburg, Germany). After centrifugation lysates were separated within NuPAGE® 4–12 % Bis-Tris Gels (Invitrogen, Carlsbad, California, USA) under reducing conditions and transferred to a nitrocellulose membrane using the iBlot® 2 dry blotting system (Invitrogen). Afterwards, iBind™ Flex FD Solution (Invitrogen) was used for blocking as well as antibody incubation applied via the iBind™ Western System (Invitrogen). The antibodies used for heavy chain (HC, #ab98616, abcam, Cambridge, UK) as well as light chain (LC, #K4377, Sigma Aldrich, St. Louis, MO, USA) detection were diluted 1:4000 and 1:8000, respectively. The loading control antibody against β-actin (#ab8227, abcam) was diluted 1:2000. Secondary IRDye® 800CW-labeled goat anti-mouse (#926-32210) as well as the IRDye® 680RD-labeled goat anti-rabbit (#926-68071) antibodies were diluted 1:4000 and the IRDye®800CW-labeled donkey anti-goat antibody (#926-32214) was diluted 1:2000 (LI-COR, Lincoln, NE, USA). Membranes were analysed using the Odyssey Infrared Scanning System (LI-COR).

2.6. Immunocytochemistry for confocal microscopic analysis

For microscopic analyses 1.7×10^4 cells/cm^2 were seeded into Nunc™ Lab-Tek™ II Chamber Slides™ (Nunc, Roskilde, Denmark) in 420 µl/cm^2 Ham´s F-12 Nutrient Mixture supplemented with 1 % fetal calf serum (Sigma Aldrich). Adherent growth of the suspension-adapted CHO cells was achieved after a cultivation for 72 h at 37 °C in a humidified HERAcell® incubator chamber (Heraeus, Hanau, Germany). After cultivation cells were fixed with 4 % paraformaldehyde (Alfa Aesar, Haverhill, MA, USA) and permeabilised with 0.1 % Triton X-100 (Carl-Roth, Karlsruhe, Germany). Subsequently, samples were blocked and incubated with an appropriate dilution of primary as well as secondary antibodies for 1.5 h and 1 h, respectively. Table 1 summarises