Secretion analysis of intracellular “difficult-to-express” immunoglobulin G (IgG) in Chinese hamster ovary (CHO) cells

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Abstract The Chinese hamster ovary (CHO) cell line is the most widely used host cell for therapeutic antibody production. Although its productivity has been improved by various strategies to satisfy the growing global demand, some difficult-to-express (DTE) antibodies remain at low secretion levels. To improve the production of various therapeutic antibodies, it is necessary to determine possible rate-limiting steps in DTE antibody secretion in comparison with other high IgG producers. Here, we analyzed the protein secretion process in CHO cells producing the DTE immunoglobulin G (IgG) infliximab. The results from chase assays using a translation inhibitor revealed that infliximab secretion could be nearly

completed within 2 h, at which time the cells still retained about 40% of heavy chains and 65% of light chains. Using fluorescent microscopy, we observed that these IgG chains remained in the endoplasmic reticulum and Golgi apparatus. The cells inefficiently form fully assembled heterodimer IgG by making LC aggregates, which may be the most serious bottleneck in the production of DTE infliximab compared with other IgG high producers. Our study could contribute to establish the common strategy for constructing DTE high-producer cells on the basis of rate-limiting step analysis.

Keywords Animal cell culture · Chinese hamster ovary cell · Therapeutic antibody production · Difficult-to-express IgG · Protein secretion

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Introduction

The Chinese hamster ovary (CHO) cell line is a workhorse of the biopharmaceutical industry (Jayapal et al. 2007; Bandaranayake and Almo 2014; Walsh 2014; Kunert and Reinhart 2016). To satisfy growing global demands for biopharmaceuticals, especially for therapeutic antibodies (Ecker et al. 2015), additional improvement of its production efficiency is required. Cell line engineering and cell culture engineering strategies have contributed to achieving very high protein titers (e.g., ~ 10 g/L) for some recombinant

proteins (Wurm 2004; Li et al. 2010; Bandaranayake and Almo 2014; Kunert and Reinhart 2016; Takagi et al. 2017; Kuwae et al. 2018). Various approaches to host cell engineering have also been studied at the genome (Omasa 2002; Kameyama et al. 2010; Kawabe et al. 2012, 2017; Wang et al. 2017), DNA transcription (Lai et al. 2013; Kawabe et al. 2017), RNA translation (Le Fourn et al. 2014; Haryadi et al. 2015; Chng et al. 2015; Kallehauge et al. 2016), and protein modification and secretion levels (Borth et al. 2005; Tigges and Fussenegger 2006; Peng and Fussenegger 2009; Pybus et al. 2014a). Such cell modification strategies have increased the productivity of recombinant protein production. Although methods for the efficient selection of high-producing cells (high producers) have also been developed (Kawahara et al. 2002; Yoshimoto et al. 2013; Lai et al. 2013; Okumura et al. 2015; Priola et al. 2016), such cells remain challenging to obtain because the established cell engineering methods usually require costly and long-term selection of rare cells by using antibiotics. Thus, this bottleneck in the establishment of high producers needs to be addressed to improve productivity.

Since the mRNA and protein levels within cells do not necessarily correlate with a high production level, the protein secretion process has been identified as a bottleneck in the production of recombinant proteins (Dinnis and James 2005; Barnes and Dickson 2006). Previous studies have proposed various strategies to overcome this bottleneck: the regulation of endogenous proteins involved in the secretion pathway (Hansen et al. 2016; Zhou et al. 2018), such as protein chaperones, heavy chain-binding protein (BiP) (Dorner et al. 1988), and protein disulfide isomerase (PDI) (Borth et al. 2005) in the endoplasmic reticulum (ER); regulation of transcription factors involved in the unfolded protein response (Tigges and Fussenegger 2006; Ohya et al. 2008; Becker et al. 2008; Haredy et al. 2013; Nishimiya et al. 2013); and regulation of the proteins related to vesicle trafficking (Peng and Fussenegger 2009; Peng et al. 2010, 2011). Studies of the microRNA that regulate the protein secretion pathway are also emerging (Pieper et al. 2017a, b). However, these strategies are sometimes ineffective for various host cells and protein products (Davis et al. 2000; Peng et al. 2010). This may be because the bottlenecks and problems can be located at various points along the complicated secretion process

depending on the specific cells and products. In addition, the protein secretory processes in suspension-cultured cells that produce high amounts of recombinant proteins might be different from the well-studied secretory pathways.

Cell- and product-specific engineering were recently suggested as means of improving recombinant protein production (O’Callaghan et al. 2010; Pybus et al. 2014a). For the efficient production of various therapeutic antibodies, including new modalities such as bispecific antibodies, Fc-fusion proteins, single chain antibodies, and biosimilars, a constant platform should be constructed for the rapid establishment of high producers based on studies about engineered cell- and product-specific phenomena. However, little is known about the details of heterologous immunoglobulin G (IgG) secretion by suspension-cultured recombinant CHO cells. Thus, acquiring fundamental information about the heterologous IgG secretion in suspension-cultured cells may guide a more efficient and rational establishment of high-producer cells.

In our previous study, we semi-quantitatively analyzed the IgG secretion by stable CHO cells producing three different therapeutic IgG (two types of IgG1 and an IgG3) (Kaneyoshi et al. 2018a). None of these three cell lines completely secreted the synthesized IgG chains; at least 20% of either the heavy chain (HC) or light chain (LC) remained in the cells after secretion stopped. These antibody components remained mainly within the ER for several hours, indicating that the post-translational modification of proteins in the ER and/or transportation of IgG between the ER and Golgi apparatus may be bottlenecks in IgG secretion. We also established cell lines producing IgG1 in which the LC is fused with fluorescent proteins to directly investigate the secretion process; results from these cells revealed that the IgG remained in the ER and cis-Golgi (Kaneyoshi et al. 2018b). However, we did not analyze the secretion of so-called “difficult-to-express” (DTE) IgG in those works. Thus, to substantially improve the productivity of cells secreting various antibodies with low yields, a quantitative secretion analysis should be performed to determine the bottleneck(s) of DTE IgG secretion.

In this study, the secretion process of a suspension CHO cell line producing infliximab (Product name: Remicade), known as a DTE IgG (Le Fourn et al.

2014), was analyzed to investigate the problems in IgG secretion in cells cultured similarly to the industrial conditions. Infliximab is one of the top-selling therapeutic antibodies (Ecker et al. 2015). It is an IgG1 antibody with kappa light chains that are the same as the IgG1A and B in our previous study (Kaneyoshi et al. 2018a), but it has a different antigen-binding site. Recently, other researchers tried to improve the production of DTE IgG by modifying the amino acid sequences, optimizing the HC:LC transfection ratio, and co-expressing protein chaperones (Pybus et al. 2014a, b). The production of infliximab was successfully improved by the overexpression of an endogenous signal recognition particle (Le Fourn et al. 2014) and the optimization of signal peptides within the chains (Haryadi et al. 2015). However, the quantitative analysis of the infliximab secretion efficiency and discussion about the localization and assembly of retained infliximab in those studies were insufficient. The present study investigated the infliximab secretion from various aspects to identify the likely bottleneck. We quantitatively analyzed the infliximab secretion efficiency by performing chase assays using a translation inhibitor and determined the length of time before the IgG secretion reached a plateau as well as the amount of retained IgG. The bottleneck of infliximab secretion was directly analyzed by visualization of IgG retention within the intracellular organelles via immunofluorescent microscopy. Additionally, the efficiency of IgG assembly was analyzed based on size exclusion chromatography (SEC). The characteristics specific to DTE IgG-producing cells are discussed in comparison with CHO cells producing other IgG that were investigated in previous studies to provide insight for constructing high producers of DTE IgG.

Materials and methods

IgG-producing cells

A CHO-Iflx suspension cell line producing the DTE antibody infliximab was established as previously described with slight modifications (Onitsuka and Omasa 2015). In brief, CHO-K1 cells were transfected with a Mammalian PowerExpress system (Toyobo, Otsu, Japan) including infliximab HC and LC genes at a 1:1 ratio using X-tremeGENE9 DNA Transfection

Reagent (Roche, Mannheim, Germany). A single clone was selected from the constructed cell pool by ClonePix 2 System (Molecular Devices, San Jose, CA, USA) and adapted to serum-free medium. The resulting established clone was cultured in 125-ml Erlenmeyer flasks (Corning, Corning, NY, USA) with 20 ml of BalanCD CHO Growth A medium (Irvine Scientific, Santa Ana, CA, USA) and 6 mM L-glutamine at 37 °C, 5% CO₂, 80% humidity, and 90 rpm.

Chase assay

Chase assays were performed as previously described (Kaneyoshi et al. 2018a). In brief, the CHO-Iflx cells were inoculated at $1\text{--}2 \times 10^6$ cells/ml and batch cultured with medium containing 50 µg/ml of the translation inhibitor cycloheximide (CHX; Nacalai Tesque, Kyoto, Japan) for 8 h. Supernatant and cell pellet samples were collected every 2 h. Cell lysate samples were prepared by suspending the cell pellet samples with tritonX-100 and sodium deoxycholate-based radio-immunoprecipitation assay (RIPA) buffer containing a 1% protease inhibitor cocktail. To investigate the changes in the amount of intracellular IgG aggregates, the cell debris insoluble in RIPA buffer was dissolved in a kind of urea-Laemmli buffer [62.5 mM Tris-HCl (pH = 6.8), 2% (w/v) SDS, 8 M urea, 5% (w/v) sucrose, modified from Le Fourn et al. 2014]. Quantitative analyses of the IgG secreted into the medium and the retained IgG were achieved by SDS-PAGE followed by western blotting. SDS-PAGE was performed under reducing conditions with a 12% running gel after normalization of the loaded cell numbers by measuring the total protein amount. Separated proteins were blotted onto polyvinylidene difluoride membranes and blocked with 5% skim milk/PBST. Infliximab on the membrane was detected by anti-human IgG conjugated with horseradish peroxidase (HRP; Abcam, Cambridge, UK) under LuminographI (ATTO, Tokyo, Japan). β-actin was detected by anti-β-actin mouse antibody (Cell Signaling Technology, Danvers, MA, USA) and anti-mouse antibody conjugated with HRP (Abcam) to correct intracellular IgG band intensities. The changes in IgG amount over time were calculated from the IgG band intensities measured by CSAnalyzer4 software (ATTO).

Immunofluorescent microscopy

Localization of the retained IgG was analyzed by observation under a fluorescent microscope as described previously (Kaneyoshi et al. 2018a). Suspension-cultured CHO-Iflx cells were sub-cultured onto poly-L-lysine-coated coverslips in a 12-well plate with Iscove's modified Dulbecco's medium (IMDM) containing 10% FBS at 37 °C, 5% CO₂ for two nights. After replacing the medium with fresh serum-free IMDM, IgG synthesis was halted by treatment with 50 µg/ml CHX for 4 h. After fixation with 4% paraformaldehyde, permeabilization with 0.1% tritonX-100, and blocking with 5% bovine serum albumin, the cells were then incubated overnight with anti-GM-130 mouse antibody (BD Biosciences, San Jose, CA, USA). Infliximab and anti-GM-130 antibody were detected by Alexa Fluor® 488-conjugated goat anti-human IgG (Life Technologies, Eugene, OR, USA) and Alexa Fluor® 647-conjugated goat anti-mouse IgG (Abcam), respectively. Staining of the ER and nucleus was performed after secondary antibody incubation using an ER-ID Red assay kit (ENZO Life Sciences, Farmingdale, NY, USA) and 4',6-diamidino-2-phenylidole (DAPI), respectively. A BZ-X710 (Keyence, Osaka, Japan) equipped with a CFI Plan Apo λ 100 × H lens (Nikon, Tokyo, Japan) and four fluorescent filters (DAPI: excitation = 360 nm, emission = 460 nm; GFP: excitation = 470 nm, emission = 525 nm; TexasRed: excitation = 560 nm, emission = 630 nm; Cy5: excitation = 620 nm, emission = 700 nm) was used for observation. Pseudo-coloring and contrast normalization were performed using ImageJ software (<https://imagej.nih.gov/ij/>). The percentage of the cells showing IgG co-localized with cis-Golgi was calculated by checking 10 images after normalization of the image contrast for each channel.

Size exclusion chromatography (SEC)

Assembly efficiency was analyzed by SEC as described previously with slight modifications (Kaneyoshi et al. 2018a). In brief, proper number of CHO-Iflx cells were collected after several hours of CHX treatment and suspended with tritonX-100-based SEC buffer. Each sample was injected into an ÄKTA Prime Plus that was equipped with a Superose™ 6 10/300 GL column (GE Healthcare Bio-

Science AB, Uppsala, Sweden). Samples were fractionated every 500 µl, and the antibodies in the odd-numbered fractions (24 fractions, excluding fraction #1) were detected by SDS-PAGE followed by western blot as described above.

Results and discussion

The secretion of DTE IgG stopped earlier compared with that of other IgG

The secretion process of the DTE IgG infliximab was semi-quantitatively analyzed via chase assay. Halting nascent IgG polypeptide synthesis by CHX enabled us to analyze the secretion percentage of IgG molecules after their synthesis in the ER. We performed western blots to investigate the changes in IgG amount because this technique allowed us to assess the secretion percentages of the HC and LC separately. The IgG secreted into the medium and the retained IgG were each detected every 2 h. The band intensities of both the HC and LC from the supernatants of control cells, which were cultured with CHX-free medium, increased with the length of incubation time, but the LC bands were very faint compared with the HC bands (Fig. 1a). In contrast, the band intensities of the HC and LC retained in the cells did not significantly change during the 8-h experimental period. When cells were treated with CHX, only very faint HC and LC bands were seen in the supernatant samples. Although the intensity of the HC bands from the cell lysates significantly decreased over time, the HC and LC retained within the cells never fully disappeared within the 8-h experimental period.

Changes in the amount of IgG chains secreted into the supernatant (Fig. 1b) and of the retained IgG chains (Fig. 1c) were determined using the band intensities from the western blots. The percentages were calculated by adjusting the 8-h band intensities of each chain in the supernatant samples of non-treated cells to 100% (Fig. 1b). For the retained IgG, the band intensities at 0 h for each sample were adjusted to 100% (Fig. 1c). When the cells were treated with CHX, the HC and LC levels in the supernatant hardly changed between 2 h and 8 h after CHX treatment. Within 2 h after CHX treatment, the secretion from CHO-Iflx cells was nearly stopped. In contrast, previous analyses on CHO cell lines producing other

Fig. 1 Chase assay of CHO cells producing a representative DTE IgG, infliximab. **a–c** The CHO-Iflx cells were batch-cultured with and without CHX, after which the IgG secreted to the medium and the retained IgG were each detected by western blot (**a**). The changes in both the HC (squares) and LC (triangles) over time in the secreted (**b**) and retained (**c**) IgG were analyzed by measuring the band intensities of luminescent

images. The IgG band intensity percentages in the non-treated and CHX-treated samples are indicated by closed and opened symbols, respectively (**b** and **c**). The band intensities of retained IgG were corrected by those of β -actin in advance. In CHX-treated cells, the secretion of IgG plateaued within 2 h (**b**), although more than 40% of HC and 65% of LC remained in the cells at this time (**c**) ($N = 3$)

IgG (Kaneyoshi et al. 2018a) showed that the secretion reached a plateau at around 4–6 h after the addition of CHX. Notably, CHO-Iflx cells seemed to hold fewer IgG chains ready to be secreted compared with other IgG-producer cells. Infliximab belongs to IgG1 and has HC and LC constant regions that are nearly the same as those of the IgG1A and B antibodies in our previous report (Kaneyoshi et al. 2018a). Despite the only difference among these antibodies being the sequence of the antigen binding site, their secretion percentages were much different from each other.

When the cells stopped secreting IgG after ~ 2 h, more than 40% of the HC and 65% of the LC remained within the cells (Fig. 1c). The amount of retained IgG in the cells continued to decrease even after the secretion stopped, which might be due to IgG degradation in the cells, and it finally reached a plateau at ~ 6 h after CHX treatment. About 25% of the HC and 40% of the LC were never secreted to the medium. The $t_{1/2}$, i.e., the length of time until the amount of target peptides decreased to $< 50\%$, of the intracellular HC and LC were calculated as < 2 h and 4 h, respectively (Fig. 1c). The shortage of HC in the CHO-Iflx cells may be one of the reasons for their low

IgG production level. Interestingly, the percentage of retained IgG chains in CHX-treated CHO-Iflx cells was similar to that in other cell lines; at least 20% of either HC or LC remained in the cells for several hours. The percentage of IgG retention seemed to be independent from the structure of the product IgG. In contrast, non-treated CHO-Iflx cells accumulated a high level of LC. The percentage of LC in non-treated CHO-Iflx cells increased linearly over an 8-h period, finally reaching about 150% (Fig. 1c), which is higher than that in other IgG-producer cells [e.g., $\sim 125\%$, $\sim 100\%$, and $\sim 130\%$ of LC in CHO-IgG1A, CHO-IgG1B, and CHO-IgG3 cells, respectively (Kaneyoshi et al. 2018a)]. This accumulation of LC in CHO-Iflx cells suggests that the synthesized chains in these cells are not ready to be secreted.

DTE IgG stayed in both the ER and cis-Golgi

We analyzed the localization of the retained IgG in CHO-Iflx cells. Fluorescent microscopy revealed that the IgG was widely spread inside the non-treated cells, forming a complicated network of tubular structures as shown in previous reports (Fig. 2, left panels)

Fig. 2 Fluorescent microscopy of infliximab-producing CHO cells. CHO-Iflx cells were observed with (right panels) and without (left panels) CHX treatment. The ER was directly stained with ER-ID (upper panels). The location of the cis-Golgi was observed via immunostaining against GM-130, a cis-Golgi-localizing protein. Pseudo-coloring was applied as follows: green, IgG; red, ER (upper panels) or cis-Golgi (lower panels);

and blue, nucleus. IgG occasionally co-localized with fluorescent signals in the ER (upper left, arrows) but more frequently with the cis-Golgi (lower panels, arrowheads). Some cells showed almost no IgG signal after CHX treatment (upper right, dotted arrows). The image contrast was optimized for clarity. Scale bars: 10 μ m. (Color figure online)

(Hasegawa et al. 2011; Mathias et al. 2018). These IgG overlapped with the ER (Fig. 2, upper left). Many cells showed IgG-dense spots near the nucleus (Fig. 2, left panels, arrows and arrowheads). This may indicate that the IgG co-localize with Russell bodies or the Golgi-apparatus. A Russell body is an aggregate of immunoglobulin in the ER that can be observed as a dense spot with a high amount of ER-resident proteins in low-secreting cells (Stoops et al. 2012; Hasegawa et al. 2014, 2017). These spots occasionally co-localized with similar spots seen in the fluorescent channel for the detection of the ER (Fig. 2, upper left, arrows). Stoops et al. (2012) reported that the Russell bodies in transiently transfected low-producer CHO cells were seen mostly in the perinuclear region rather than being juxtanuclear globular structures; we observed some small spots beside the nucleus in our stably transfected CHO cells. However, we more frequently observed the IgG in our cells as accumulated in perinuclear structures with complicated morphology that significantly overlapped with the signals from GM-130, a cis-Golgi marker (Fig. 2, bottom left, arrowheads). Although it is difficult to accurately determine

the precise localization of IgG from microscopy images, we concluded that the IgG likely accumulates in the Golgi apparatus rather than in Russell bodies. After image contrast normalization, the percentage of cells showing IgG as co-localized with cis-Golgi was calculated as at least 61%. This percentage is higher than that of other CHO cell lines [52.0%, 17.3%, and 47.0% for CHO-IgG1A, CHO-IgG1B, and CHO-IgG3, respectively (Kaneyoshi et al. 2018a)].

Our analysis of CHO-Iflx cells that were treated with CHX for 4 h produced very faint IgG signals. This is understandable, given that the chase assay showed that only 30% of the HC and 50% of the LC remained in the cells after a 4-h CHX treatment (Fig. 1c). We found that the IgG were still widely spread inside the cells, roughly overlapping with the ER (Fig. 2, upper right), and these retained IgG produced dot-like signals instead of the complicated network seen in non-treated cells. The intensity of IgG signals differed from cell to cell, suggesting that the secretion efficiency varies among individual cells. Notably, cells with almost no IgG signal were frequently observed (Fig. 2 upper right, dotted

arrows). IgG remained in the cis-Golgi even 4 h after the addition of CHX (Fig. 2, bottom right, arrow-heads). Although it was quite difficult to determine the percent of IgG co-localized within the cis-Golgi due to the faint signals from the IgG, at least 38% of cells showed co-localization. This percentage was higher than that of CHO-IgG1 cells but lower than that of CHO-IgG3 cells from our previous study (24.2%, 11.0%, and 84.5% for CHO-IgG1A, CHO-IgG1B, and CHO-IgG3, respectively, Kaneyoshi et al. 2018a).

Together, these results indicate that the infliximab stayed in the ER and cis-Golgi for a long time even after the secretion plateaued. Thus, the bottleneck in the process of infliximab secretion occurs in the ER and cis-Golgi, during the early steps of the secretion process. To our knowledge, this is the first report investigating the localization of retained infliximab in stably infliximab-expressing CHO cells. Stoops et al. (2012) reported that IgG stayed in the ER forming Russell bodies in transiently transfected, poorly secreting HEK293 and CHO cells. Another study showed that a DTE bispecific antibody was not detectable in the cis-Golgi in stable recombinant CHO cells, even though a considerable amount of normal recombinant IgG is co-localized with the cis-Golgi in high-producer CHO cells (Mathias et al. 2018). In contrast, our results show that in CHO cells stably producing infliximab, a representative DTE IgG, the IgG co-localized with the cis-Golgi more frequently compared with that in other IgG producers, so the infliximab-producing cells may have another limiting step in the Golgi apparatus. Thus, there does not seem to be a single bottleneck that limits all DTE antibodies.

Infliximab in CHO-Iflx cells assembled into heterodimer IgG less efficiently compared with other IgG

Because fluorescent microscopy revealed that infliximab remained in the ER for several hours, we used SEC to further analyze the IgG assembly. IgG HC and LC polypeptides are first folded, and then the properly-folded HC and LC are assembled into heterodimer IgG in the ER (Haredy et al. 2013). Misfolded proteins may cause aggregation. After glycosylation, the IgG can be transported from the ER to the Golgi apparatus. The retention of infliximab in the ER may indicate that the HC and LC expressed in CHO cells have problems

with assembly. After separating intracellular proteins by SEC under non-reducing conditions, the amounts of HC and LC in each fraction were analyzed by western blot under reducing conditions. We chose to perform this quantification by western blotting rather than by UV absorption because western blotting, unlike UV absorption, does not require purification steps with IgG affinity columns such as Protein A and G, which may not recover aggregates and immature forms of IgG.

In cells that were not treated with CHX, the HC and LC bands were mainly in the second membrane (Fig. 3a, elution volume 13–24 ml). The largest HC peak was around 16 ml, indicating that most HC is assembled into an IgG molecule (Fig. 3b, black arrow). In these fractions, a LC peak was also seen as expected. HC and LC also each showed a shoulder peak around 13 ml, possibly revealing that a subset of fully assembled IgG formed oligomers or aggregates. There was a total of three LC peaks, the largest of which was observed at around 20 ml (Fig. 3b, arrow-head). Almost no HC was found around these fractions, suggesting that most of the LC population accumulates as free chains without interacting with the HC. This finding differs from previous reports showing almost no free-LC in the tritonX-100-soluble fraction (Le Fourn et al. 2014). Only 26% of LC likely assembles with HC (at 16 ml) as compared with free-LC (set to 100% at 20 ml). After CHX treatment for 1 h or 4 h, the intracellular IgG was fractionated as well (Fig. 3c and e). Regardless of the CHX treatment duration, a single HC peak was seen at 16 ml, and no peak corresponding to free-HC was found (Fig. 3d and f, black arrows). When the cells were treated with CHX for 1 h, the LC peak around 16 ml decreased (Fig. 3d, black arrow), possibly due to the secretion or degradation of fully assembled IgG, and the largest LC peak was still at 20 ml, suggesting that much of the free-LC still remained in the cells without interacting with the HC. However, a peak was also observed at 8 ml, indicating the formation of LC aggregates. Only a LC band was observed in this fraction, so the LC may form aggregates before assembling with the HC (Fig. 3c, white arrow). Although the total amount of LC significantly decreased during the CHX treatment (Fig. 1c), a higher percentage of LC may form aggregates rather than assemble into heterodimer IgG molecules. When the cells were treated with CHX for 4 h, it was almost impossible to detect LC

Fig. 3 Size exclusion chromatography analysis of intracellular IgG. **a–f** CHO-IgG1A cell lysate samples taken 0 h (**a** and **b**), 1 h (**c** and **d**), and 4 h (**e** and **f**) after the addition of CHX were collected and separated by SEC. The IgG in the fractionated samples were detected by western blot (**a**, **c**, and **e**) and the band intensity ratios of HC (Closed squares) and LC (opened triangles) were analyzed (**b**, **d**, and **f**). As LC bands were very faint when the cells were treated with CHX for 4 h (**e**), the LC band intensities were not analyzed in this sample (**f**). The exposure time for detection of luminescence was optimized to

each sample, and the contrast was optimized for clarity (**a**, **c**, and **e**). An open arrow in (**c**) indicates the LC aggregation. The band intensity ratio was calculated by adjusting the intensity of the strongest peak in each chain to 100% (**b**, **d**, and **f**). Although the HC showed a significant peak at the same elution volume regardless of the length of CHX treatment (**b**, **d**, and **f**, black arrows), the LC showed many peaks. The strongest LC peak was observed at around 20 ml, indicating free-LC that had not assembled with the HC (**b** and **d**, arrowheads)

bands on the membranes though the HC bands were significantly observed (Fig. 3e). Although this might be caused from insufficient exposure time, the band intensity of LC was not analyzed to avoid misleading conclusions (Fig. 3f).

Infliximab from CHO-IgG1A showed inefficient assembly into IgG molecules, unlike the IgG1A from

the CHO-IgG1A cells generated in our previous study (Kaneyoshi et al. 2018a). The HC and LC in CHO-IgG1A each showed a significant peak at 16 ml, even several hours after CHX treatment, suggesting that the assembly of HC and LC was almost complete. Although the CHO-IgG1A cells finally retained about 50% of the HC and 25% of the LC, the bottleneck in

IgG1A secretion appeared to occur after the assembly of HC and LC. Based on this comparison with the CHO-IgG1A cells (Kaneyoshi et al. 2018a, Fig. 4a, c), there are three possible explanations for the lower secretion level of infliximab (Fig. 4b, d).

First, a constant amount of IgG remained in the CHO-Iflx cells even after IgG assembly (Fig. 4(1)); the largest HC peak was always observed at 16 ml, but only very faint LC bands were found in these fractions. This result could be caused by the amount of LC assembled with HC being lower than the detection limit of the western blot, a possibility that is supported by the finding that the band intensities of LC were smaller than those of HC in the culture supernatant even though there should be the same number of both chains (Fig. 1a). Additionally, a non-reducing western blot in a previous study showed that infliximab-producing cells retained fully assembled IgG even 6 h after the addition of CHX (Le Fourn et al. 2014). Notably, this explanation does not conflict with the fact that a constant amount of IgG remained in the cis-Golgi even after CHX treatment because IgG HC and LC are regulated to be transported to the cis-Golgi after assembly. This point is not critical though,

because CHO cells producing IgG1A showed a lower but similar percentage of IgG co-localization with the cis-Golgi as that in CHO-Iflx cells (For CHO-IgG1A cells, 52.0% and 24.2% before and after CHX treatment, respectively (Kaneyoshi et al. 2018a), and for CHO-Iflx cells, at least 61% and 38% before and after CHX treatment, respectively).

The second possible explanation is an imbalance between the HC and LC polypeptides (Fig. 4(2)). If the LC is overexpressed compared with the HC, the excess LC would not have a chance to assemble into a single IgG molecule due to HC unavailability. This possibility is feasible given that the HC level decreased earlier than the LC level (Fig. 1c). It is also supported by the higher LC band intensities from the cells compared with the HC band intensities from these samples (Fig. 1a). Notably, the intracellular HC also accumulated during cell culture without CHX (Fig. 1c), indicating that there are additional causes besides a shortage of HC for the inefficient secretion of infliximab. In addition, such imbalance in HC and LC polypeptides was also found in other IgG producers (Kaneyoshi et al. 2018a), thus this may not be critical to explain the inefficient secretion of DTE infliximab.

Fig. 4 Schematic diagram showing possible limiting steps in the secretion of IgG. **a–d** The HC and LC of a model easy-to-express IgG1A (Kaneyoshi et al. 2018a) efficiently assembled into heterodimer IgG (**a**) even after CHX treatment (**c**); the bottleneck in this process seems to be in the posttranslational modification after assembly. There are three possible limiting steps in the infliximab-producing cells as revealed by their comparison with native (**b**) and CHX-treated (**d**) IgG-secreting cells: (1) retention of fully assembled IgG in the Golgi apparatus, (2) shortage of HC compared with LC, and (3) misfolding and aggregation of the LC without assembly with the HC

The third possibility is that the LC undergoes misfolding and aggregation such that the LC is unavailable for IgG assembly (Fig. 4(3)). Based on our SEC results, this may be the most serious bottleneck in the infliximab secretion process. The peak intensity of LC aggregation (at 8 ml) was larger than that of LC in the fully assembled IgG fraction (at 16 ml) when the cells were treated with CHX for 1 h (Fig. 3c, d). Further investigation into aggregation formation was performed using a solubilization buffer with urea and SDS (modified from Le Fourn et al. (2014)) to extract proteins from the debris after a mild protein extraction with RIPA buffer. The chase assay of these tritonX-100 insoluble fractions did not show any HC, whereas they showed an almost constant amount of LC; however, both the HC and LC levels in the tritonX-100-soluble fractions significantly decreased after CHX treatment (Fig. 5). Insoluble LC remained in cells even after 8 h of CHX treatment. The existence of LC aggregates in tritonX-100-insoluble fractions has been reported by Le Fourn et al. (2014). Additionally, Stoops et al. (2012) showed that, when is expressed alone, LC in low-producer cells forms Russell bodies but not in high-producer cells, and they concluded that the LC plays an important role in determining the secretion efficiency.

Fig. 5 A chase assay using tritonX-100-soluble and -insoluble fractions. The tritonX-100-insoluble samples were collected by resuspending cell debris after RIPA buffer extraction

However, our microscopic observation did not show a significant retention of IgG aggregates in Russell bodies. While this explanation seems to be the most likely based on our data, it is difficult to conclusively determine only one bottleneck in the process of DTE IgG secretion.

From our results, improvement in the HC:LC ratio via overexpression of HC or suppression of LC aggregation and downstream transportation from the Golgi apparatus may be effective at increasing the productivity in CHO-Iflx cells. The differences in the CHO-Iflx secretion from the secretion in other IgG producers suggests that cell-, clone-, or product-specific strategies will need to be developed based on quantitative secretion analyses of each cell line. For example, even though both studies used CHO cells producing infliximab, Le Fourn et al. (2014) reported that tritonX-100-soluble free-LC in the cells was undetectable by western blotting, whereas our SEC analysis showed the existence of free-LC in the tritonX-100-based SEC buffer. In addition, it would be valuable to compare the IgG expression levels in each cell line by quantitative reverse transcription PCR (qRT-PCR), though we did not perform it here.

In summary, we determined the secretion efficiency and possible rate-limiting steps of CHO cells producing the DTE IgG infliximab. As with other IgG-producing cell lines, CHO-Iflx cells retained more than 20% of synthesized IgG chains. Interestingly, at least 20% of HC or LC remained in the cells regardless of the IgG product type. Our SEC analysis revealed that the infliximab LC and HC undergo inefficient assembly. However, our immunofluorescent microscopic observations found CHO-Iflx cells with IgG colocalization and retention within the cis-Golgi, indicating that there may be another bottleneck in the Golgi apparatus. The future application of the relatively easy analyses used in our study may guide the development of common strategies for improving inefficient secretion of other DTE IgG.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This manuscript does not contain information about the research involving human participants and/or animals

Informed consent Informed consent is not necessary because we do not report a study involving human participants and/or animals.

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