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Analysis of intracellular IgG secretion in Chinese hamster ovary cells to improve IgG production

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The production of biopharmaceutical immunoglobulin G (IgG) using cultured mammalian cells, especially Chinese hamster ovary (CHO) cells is well established and has been markedly improved through the modification of cells and cell culture engineering technologies. The establishment of high-production cell lines remains a challenge. The intracellular secretion of IgG has been investigated to identify and solve the rate-limiting steps in antibody production. However, strategies that regulate the expression of proteins that are related to antibody secretory pathway have not consistently improved their production. In this study, key features and limitations of the antibody secretion process in recombinant CHO cells were analyzed to develop more efficient approaches for establishing high-production cells. By chase assay with protein translation inhibitors, IgG secretion reached a plateau when at least 20% of IgG remained in the cells. The secretion kinetics and retention ratio of IgG varied between IgG subclasses (two types of IgG1 and an IgG3 subclass). Immunofluorescent microscopy and size exclusion chromatography showed that the remaining intracellular IgG localized mainly within the endoplasmic reticulum (ER) and less with the cis-Golgi network, despite the formation of fully assembled IgG. These results show that remaining intracellular IgG is a target for enhancing antibody secretion, even in high-production CHO cells.

[**Key words:** CHO cell; Animal cell culture; Therapeutic antibody; Recombinant protein production; Intracellular protein secretion]

As the market for biopharmaceutical monoclonal antibody products continues to grow (1), further development of production processes using mammalian cells—typically, Chinese hamster ovary (CHO) cells—is required to reduce production costs and ensure product quality. Previous studies have established high-production cells through pre-translational modifications (2), such as amplification of gene copy numbers (3–8) and improvement of transcription efficiency (9), resulting in very high titers, in combination with improved culture methods (10–14). A method to select high producers has also been developed (15). However establishing high producers still remains a challenge, because the typical method requires long-term selection and cell line-specific optimization of culture processes.

Post-translational processes are bottlenecks in protein production, consistent with the finding that higher mRNA levels do not correlate with increased protein synthesis (2,16). To overcome the secretory bottleneck, studies from the past two decades have examined the up- and downregulation of proteins in the secretory pathway. For instance, overexpression of protein disulfide isomerase (PDI) (17,18) and the Sly1, Munc18b, and soluble N-ethylmaleimide-sensitive factor receptor (SNARE) proteins (19–21) has improved recombinant protein production. The down-regulation of immunoglobulin heavy-chain binding protein (BiP) has improved heterologous protein production (22). Over-expression of transcription factors that are involved in the unfolded protein response (UPR) (23), such as X-box binding proteins (XBP1) (24–27), activating transcription factor (ATF) (28,29), and CCAAT-enhancer-binding protein homologous protein (CHOP) (30), to target protein secretion and cell proliferation has also enhanced protein production. These studies demonstrate that intracellular secretion is a bottleneck in recombinant protein production. However, these strategies do not always improve protein production, which is dependent on the target products and host cells (20,31), and recent studies have suggested that product- and cell line-specific engineering strategies are necessary to improve production (31,32).

There is no single or simple strategy to improve the secretion of recombinant proteins, perhaps because of the complicated network of secretory and signaling pathways. The efficient establishment of high producers for existing and emerging biopharmaceuticals requires further rational cell engineering that is based on a quantitative investigation of secretion in engineered cells that have been modified to produce non-native recombinant proteins in serum-free media.

Previous studies have repeatedly shown that various types of cells do not secrete all of the synthesized proteins into the medium

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and retain their proteins for several hours (33–35). However, they did not perform a quantitative analysis of secreted and remaining proteins among CHO cells producing various types of immunoglobulin G (IgG), and there are limited analyses of such remaining proteins. Investigations of remaining proteins can guide the more efficient use of synthesized polypeptides, which may lead to further improvements in the production of high-production cells.

In this study, the duration of intracellular secretion and the amount of IgG that was retained in CHO cells were investigated by chase assay using translation inhibitors, and cells producing various types of IgG were compared. Characteristics that impacted IgG secretion in these CHO cells are discussed, including localization and maturation of such remaining IgG.

MATERIALS AND METHODS

IgG-producing cells CHO cells producing three types of therapeutic IgG [two types of IgG1 and an IgG3, named CHO-IgG1A (36), CHO-IgG1B, and CHO-IgG3 (37), respectively] were used. IgG1A and 1B have a common constant region but different variable region. IgG1A and 1B heavy chain (HC) and light chain (LC) genes were integrated into the Mammalian PowerExpress System (Toyobo, Otsu, Japan) at a 1:1 ratio, and the constructed plasmid was transfected into CHO–K1 cells using the X-tremeGENE9 DNA Transfection Reagent (Roche, Mannheim, Germany) (36). The IgG3 expression plasmid (37) was transfected as described for the IgG1-producing cells.

Chase assay for intracellular IgG CHO-IgG1A and CHO-IgG3 cells were cultured in in-house serum-free medium [a mixture of TOp2 (Irvine Scientific, Costa Mesa, CA, USA) and EX-CELL CD Hydrolysate Fusion (Sigma Aldrich, St. Louis, MO, USA) for CHO-IgG1A and a mixture of BalanCD CHO Growth A (Irvine Scientific), EX-CELL CD Hydrolysate Fusion, and Hyclone CDM4 CHO (GE Healthcare, Buckinghamshire, UK) for CHO-IgG3]. CHO-IgG1B cells were cultured in BalanCD CHO Growth A. Translation inhibitor cycloheximide (CHX) was purchased from Nacalai Tesque (Kyoto, Japan). The powder of cycloheximide was dissolved in ethanol at a concentration of 10 mg/ml as stock solution. The three types of CHO cells were seeded at 2×10^6 cells/ml and cultured with 20 ml of media containing 6 mM L-glutamine and 50 μg/ml of CHX in Erlenmeyer flasks at 37°C, 5% CO_2, 80% humidity, and 90 rpm on a Climo-Shaker ISF1-XC (Kuhner, Birsfelden, Switzerland). One milliliter of culture was collected every 2 h, and the supernatant samples were collected after centrifugation. After the cell pellets were washed with PBS, cell lysate samples were obtained by suspending the pellets in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.5% (w/v) sodium deoxycholic acid, 0.5% (v/v) TritonX-100] with 1% protease inhibitor cocktail (Nacalai Tesque), vortexing them, and removing the insoluble debris by centrifugation.

SDS-PAGE and quantitative western blot To normalize the cell numbers that were loaded, the total protein content in each lysate sample was measured using a BCA protein assay kit (ThermoFisher Scientific, Rockford, IL, USA). After adjustments of the protein amount by dilution in MilliQ water, the samples were mixed with 4 × Laemmli buffer (250 mM Tris-HCl (pH 6.8), 20% 2-mercaptoethanol, 8% SDS, 20% sucrose, and 0.02% BPB). The supernatant samples were mixed with 4 × Laemmli buffer without dilution. Both the lysate and supernatant samples were boiled for 5 min, and loaded onto polyacrylamide gels with parts including a running gel (375 mM Tris-HCl (pH 8.8), 12% acrylamide-bis solution, 250 mM sucrose, 0.1% SDS) and a stacking gel (125 mM Tris-HCl (pH 6.8), 4% acrylamide-bis solution, 1% SDS).

After the electrophoresis, the separated proteins in the gel were blotted onto a polyvinylidene difluoride membrane (Immobilon-P, Merck Millipore, Carrigtwohill, Ireland) using semi-dry blotting devices. The membranes were incubated with blocking buffer [5% skim milk/PBST (PBS with 0.1% Tween20)] for 1 h at room temperature (RT). For the detection of β-actin, the membranes were incubated with anti-β-actin (8H10D10) mouse mAb (#3700, Cell Signaling Technology, Danvers, MA, USA) that was diluted in 1% skim milk/PBST (1:10,000) at 4°C overnight. After being washed with PBST, the membranes were incubated with secondary antibodies [horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG H&L (ab6759) and HRP-conjugated rabbit anti-mouse IgG H&L (ab6728) from Abcam (Cambridge, UK) for the detection of recombinant IgG and anti β-actin, respectively] that were diluted in 1% skim milk/PBST (1:10,000) for 4 h at RT and then washed with PBST. The changes in the amount of target protein were detected based on luminescent intensities using chemiluminescent HRP substrate (Immobilon Western, Merck Millipore, Billerica, MA, USA), Luminograph I, and CSAnalyzer 4 software (ATTO, Tokyo, Japan).

Immunofluorescent microscopy Suspension cultured cells were passaged into 12-well plates with 1 ml IMDM (10% FBS) and poly-L-lysine-coated coverslips for two nights. The medium was changed to serum-free IMDM with 50 μg/ml CHX and incubated at 37°C, 5% CO_2 for 4 h. After being washed with PBS, the cells were fixed in 4% paraformaldehyde/PBS at RT for 15 min. The paraformaldehyde was removed by a single wash with 100 mM glycine/PBS and two wash steps with PBS. The cells were permeabilized with 0.1% TritonX-100/PBS for 5 min and washed with PBS. The cells were blocked with 5% bovine serum albumin (BSA)/PBS at RT for 1 h and incubated with primary antibodies [anti-GM-130 (35/GM130) mouse antibody (610822, BD Biosciences, San Jose, CA, USA)] that were diluted in 5% BSA/PBS (1:200) at 4°C overnight. After being washed with PBS, the cells were incubated with secondary antibodies that were diluted in 5% BSA/PBS (1:500), Alexa Fluor 647-conjugated goat anti-mouse IgG H&L (ab150119, Abcam) and Alexa Fluor 488-conjugated goat anti-human IgG (A11013, Life Technologies, OR, USA) at RT for 1 h. The cells were then counterstained with 1 μg/ml of 4',6-diamidino-2-phenylidole (DAPI) for 5 min. After a wash step with MilliQ water, coverslips were fixed on the glass slides with fluorescent mounting medium (DAKO North America, Carpinteria, CA, USA).

Detection of the ER was performed by direct staining with the ER-ID Red assay kit (ENZO Life Sciences, Farmingdale, NY, USA). Staining was performed between the secondary antibody treatment and DAPI staining by incubating the cells with ER-ID reagent/PBS (1:500) at 37°C for 15 min, followed by a wash step with PBS.

A BZ-X710 (Keyence, Osaka, Japan) that was equipped with DAPI, GFP, TexasRed, and Cy5 filters was used. Pseudocoloring and normalization of contrast were performed using ImageJ software (https://imagej.nih.gov/ij/). The co-localization of IgG within cis-Golgi was determined by microscopic observations, and the frequency was calculated by dividing the number of cells showing co-localization within cis-Golgi by the number of total cells (>80 cells).

Size exclusion chromatography Ten milliliters of cultured CHO-IgG1A cell were collected after several hours of CHX treatment. After being washed with PBS, the collected cell pellets were suspended in size exclusion chromatography (SEC) buffer [20 mM HEPES-KOH (pH 7.4), 150 mM KCl, 5% glycerol, and 0.1% TritonX-100] and vortexed, followed by removal of cell debris by centrifugation. For further removal of tiny debris, the sample was centrifuged at 10,000 × *g*, 4°C for 10 min. The 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). Fractioned samples were collected every 500 μl. The IgG in the odd-numbered fractions (25 fractions in total) was detected by western blot as described above.

RESULTS

IgG secretion in cells reached a plateau despite intracellular retention of HC and LC Temporal changes in the amount of IgG1A remaining in the cells or secreted into the medium were analyzed by inhibiting nascent peptide synthesis with CHX (Fig. 1A and B). CHX concentration was optimized to 50 μg/ml, which was comparable in performance with 100 μg/ml CHX (data not shown). During the culture with CHX, cell viability was maintained at 99%, but cell division was inhibited (data not shown). Each lane showed double bands at approximately 55 and 25 kDa, indicating the HC and LC of IgG, respectively (Fig. 1A). When the cells were cultured with CHX, the band intensity in the cell lysate decreased during the culture, especially for the LC. The band intensities of both chains in the culture medium increased during the culture, showing that the IgG that was synthesized in the cells was secreted into the medium.

The changes in the intensity of the IgG bands was analyzed after normalization to β-actin (Fig. 1B). The ratio of each chain was calculated by defining the intensity of both chains at 0 h as 100% in the analysis of the changes in intracellular IgG. For the analysis of secreted IgG, the intensity of both chains at 8 h in the samples without CHX was defined as 100%. When the cells were cultured with CHX, the HC and LC in the culture medium increased until 4 h after the inhibition, but then remained stable, unlike the controls, which increased (Fig. 1B). The ratio of secreted IgG from cells reached a plateau after approximately 4–6 h. The cells could not secrete all of the synthesized IgG into the medium, and approximately 50% of HC and 25% of LC remained in the cells for several hours.

The CHO-IgG1B and -IgG3 cells were analyzed using the same conditions as the CHO-IgG1A cells (Fig. 1C and E). Intracellular IgG1B LC decreased to 60% during the first 2 h and then gradually decreased to 40% by 8 h (Fig. 1D). The intracellular HC level was

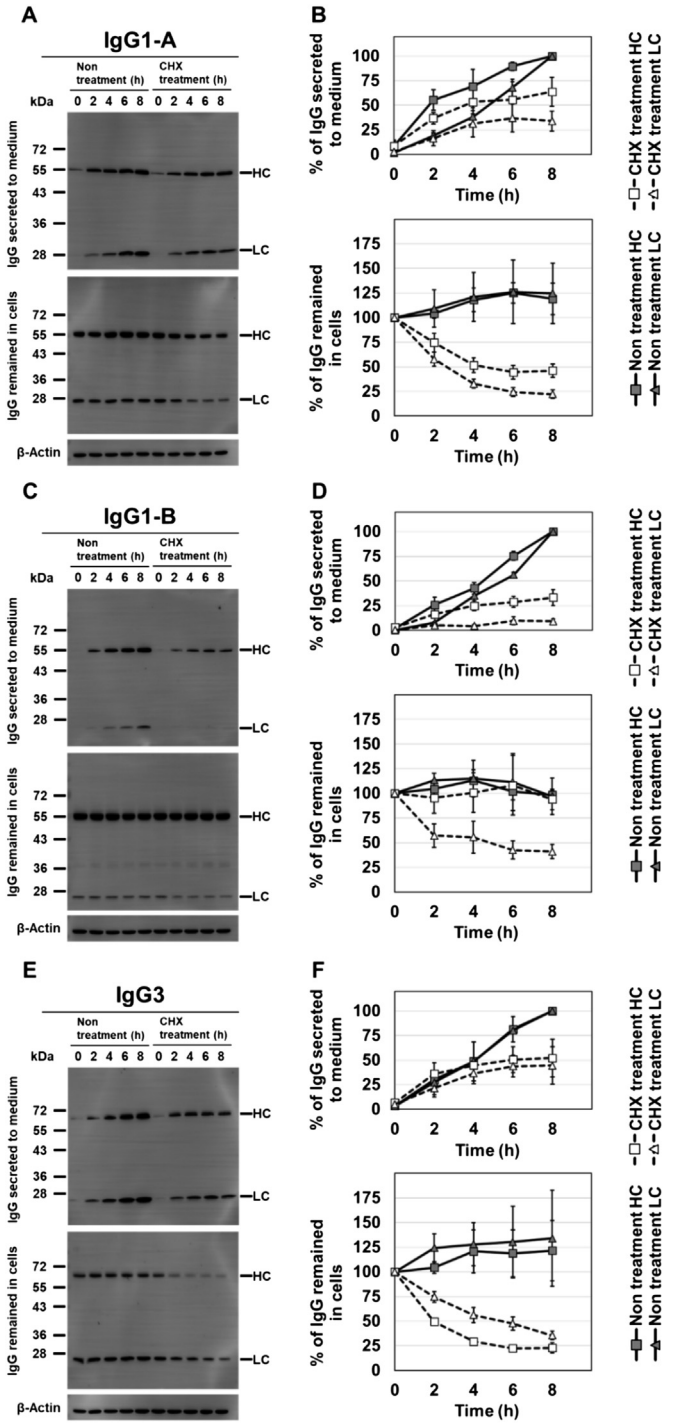

FIG. 1. Chase assay of CHO-IgG1A (A, B), CHO-IgG1B (C, D), and CHO-IgG3 cells (E, F). The immunoglobulin heavy (HC) and light (LC) chain band intensities from western blot in each cell line (A, C, E) were measured, and the changes in the ratio are shown as graphs (B, D, F). The band intensities at 0 and 8 h were defined as 100% in the graphs of the cell lysate and supernatant samples, respectively (n = 3).

relatively constant (>90%) throughout the 8-h culture, and the amount of secreted IgG stabilized at 4–6 h (Fig. 1D). Twenty-five percent of the HC and 50% of the LC remained in the CHO-IgG3 cells when the secretion reached a plateau at 4–6 h after the CHX-induced inhibition (Fig. 1F). These results indicate that the secretion of IgG was completed within several hours after the inhibition of translation, despite over 20% of the synthesized IgG chains remaining in the cells.

IgG remained primarily in the ER by immunofluorescent microscopy To investigate the characteristics of the remaining IgG, the co-localization of IgG within the ER and cis-Golgi was analyzed with and without CHX treatment. CHO-IgG1A cells that were cultured without CHX contained a considerable amount of intracellular IgG, mainly within the ER (Fig. 2, upper left) and partially within the cis-Golgi (Fig. 2, bottom left). After CHX treatment for 4 h, a reduction in intracellular IgG was observed, based on the decrease in green fluorescent intensity. After normalization of image contrast, we observed that IgG co-localized mostly within the ER (Fig. 2, upper right) and hardly within the cis-Golgi (Fig. 2, bottom right). The green fluorescent intensity in cells differed between cells, suggesting that there was a variable amount of remaining IgG between individual cells. The ratio of cells with IgG that co-localized within the cis-Golgi decreased from 52.0% to 24.2% after CHX treatment. Newly synthesized IgG appeared to remain within the ER for several hours; thus, the bottleneck in IgG secretion appears to exist in the ER.

In CHO-IgG1B cells, human IgG co-localized partially with the ER (Fig. 3, upper panels) but rarely with the cis-Golgi (Fig. 3, lower panels), regardless of CHX treatment (17.3% and 11.0% of cells showed IgG co-localizing within the cis-Golgi before and after CHX treatment). In CHO-IgG3 cells, IgG co-localized mainly with the ER (Fig. 4, upper panels) and less with the cis-Golgi (Fig. 4, lower panels), regardless of CHX treatment. Although 47.0% of cells contained IgG that co-localized with the cis-Golgi without CHX treatment, the percentage increased to 84.5% after the treatment.

The remaining intracellular IgG formed fully assembled antibody CHO-IgG1A cells were incubated with CHX for 0, 4, and 8 h, and intracellular proteins were fractionated by SEC. IgG was detected by western blot of the fractioned samples to skip the IgG purification step using affinity columns and avoid losing some of the aggregate or immature forms of IgG. Both HC and LC were eluted at a significant peak at around 16 ml, regardless of the duration of CHX treatment (Fig. 5). Because the bovine gamma globulin (158 kDa) in the gel filtration standard (Bio-Rad Laboratories, Hercules, CA, USA) eluted at 16.47 ml, this peak suggested the presence of fully assembled IgG, even after inhibition of protein synthesis by CHX for 4 h (Fig. 5A) and 8 h (Fig. 5B). Therefore, IgG was not secreted into the medium, despite the intracellular formation of fully assembled IgG. Without CHX treatment, larger HC and LC species were observed, which indicates aggregate or oligomer IgG (Fig. 5C), but not after CHX treatment. A peak of LC eluted at around 20 ml after CHX treatment for 4 h (Fig. 5A). However, it nearly disappeared by 8 h after the inhibition.

DISCUSSION

In this report, we semi-quantitatively analyzed the intracellular secretion of recombinant IgG in CHO cells and investigated the intracellular localization and maturation of IgG. A chase assay using a translation inhibitor in the cell culture was used to determine the duration of IgG secretion and the retention ratio. This basic technique showed that IgG secretion reached a plateau, even though over 20% of the intracellular IgG remained in all cells; however, the duration and retention ratio differed between cell lines expressing various types of IgG. Although IgG1A and B have a common Fc region, they exhibited a different ratio of IgG retention. In contrast, IgG3 has a longer Fc region and showed a secretion ratio that was comparable with IgG1A. Therefore, there was no correlation between the secretion ratio and the Fc structure of IgG.

For the measurement of IgG retention and secretion ratio, we performed western blotting. This technique was useful to analyze

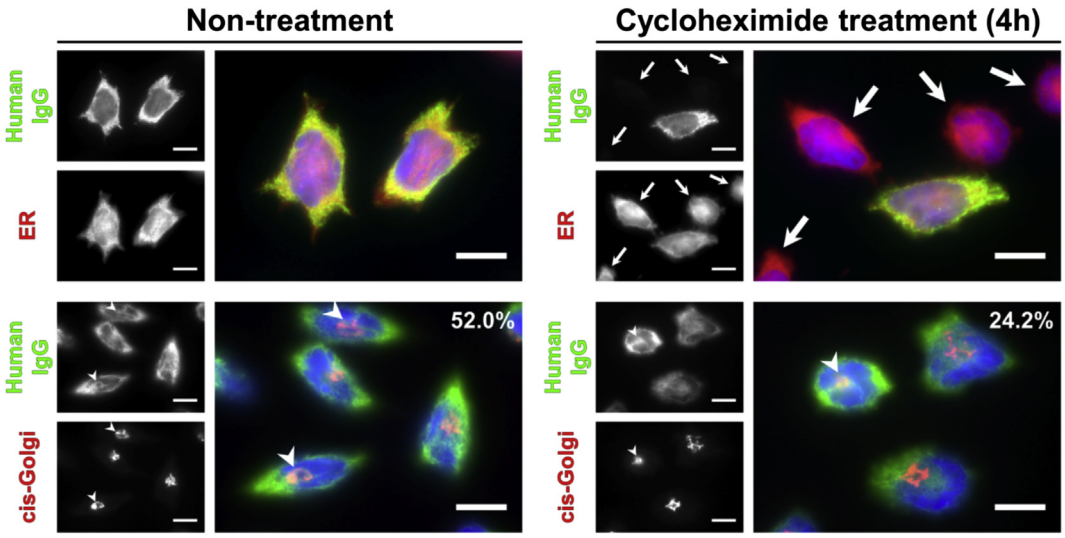

FIG. 2. Immunofluorescent microscopy of CHO-IgG1A cells. Cells were observed with (right panels) and without (left panels) cycloheximide (CHX) treatment. Pseudocoloring was performed as follows: green indicates IgG1A, red indicates the endoplasmic reticulum (ER) (stained by ER-ID, upper panels) or cis-Golgi (detected by anti-GM-130, lower panels), and blue indicates the nucleus (stained by DAPI). The intensity of green fluorescence differed between cells, and some cells showed very low fluorescent intensity (arrows). Partial co-localization of human IgG and cis-Golgi (arrowheads) was detected. The ratios of cells which human IgG co-localized within cis-Golgi are shown in the merge images (bottom panels). Bars: 10 μm.

the changes in the amount of both chains separately. However the absolute concentration of IgG could not be discussed. It may be of worth to discuss the IgG titer based on the quantification by enzyme linked immunosorbent assay (ELISA). We repeated chase assay, and the concentration of secreted IgG at each time point was analyzed by sandwich ELISA using anti-human IgG Fc antibodies. When the cells were cultured without CHX, the titer linearly increased during the cultivation. After 8 h, non-treated CHO-IgG1A, -IgG1B, and -IgG3 secreted 3.36, 3.78, and 6.29 μg/ml of IgG, respectively. On the other hand, CHX treated cells stopped the secretion 2–6 h after the addition of CHX, and CHO-IgG1A, -IgG1B, and -IgG3 secreted up to 1.08, 0.840, and 2.13 μg/ml of IgG, respectively. These results indicated that CHX treated cells secreted 20–35% of IgG by 8 h compared to non-treated cells. This ratio is slightly smaller than that calculated from the band intensities of western blotting (Fig. 1B, D, and F). Although, because it is difficult to determine the accurate concentration of IgG secreted from a small population of cells within several hours, the measurement by multiple methods might be still required.

The duration of protein secretion has been examined extensively using mammalian cells that produce native or recombinant proteins. Previous studies have reported that a eukaryotic cell takes <1 h to secrete a protein in most cases (38), and one study found the $t_{1/2}$ (the time for the amount of a target in cells to decrease by half) of INF-γ secretion from human T cells to be 20–25 min (34). In contrast, the $t_{1/2}$ of IgG secretion from plasmacytoma cells and transiently transfected HEK293 cells is 2–3 h and 8 h, respectively (33,35). However, there has been limited discussion of recombinant

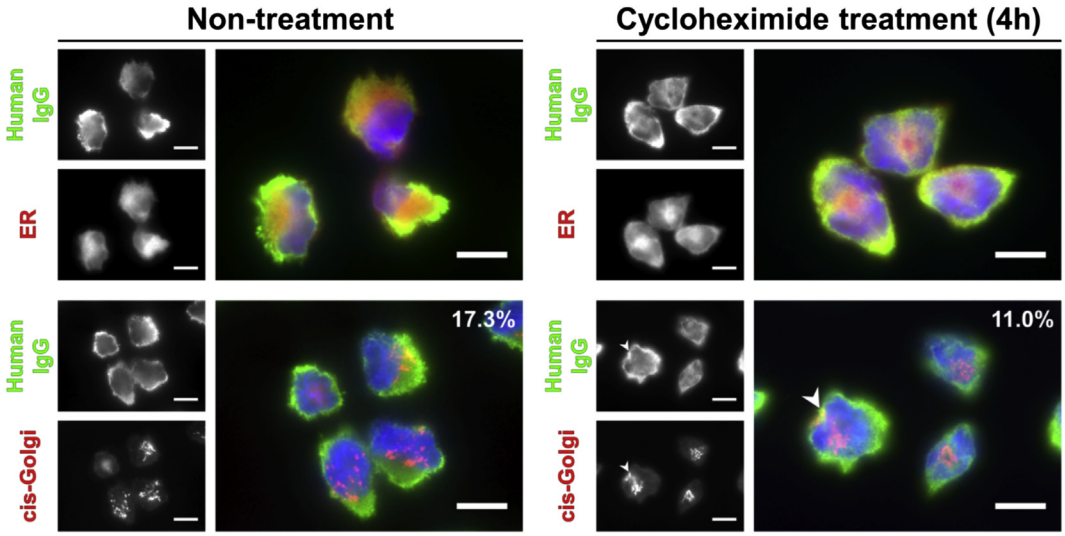

FIG. 3. Immunofluorescent microscopy of CHO-IgG1B cells. Cells were incubated for 4 h in serum-free medium with (right panels) or without (left panels) cycloheximide (CHX). Green indicates IgG1B, red indicates the endoplasmic reticulum (ER) (upper panels) or cis-Golgi (lower panels), and blue indicates the nucleus. Localization of IgG in the cis-Golgi was rarely seen (lower right panel, arrowhead). The ratios of cells which human IgG co-localized within cis-Golgi are shown in the merge images (bottom panels). Bars: 10 μm.

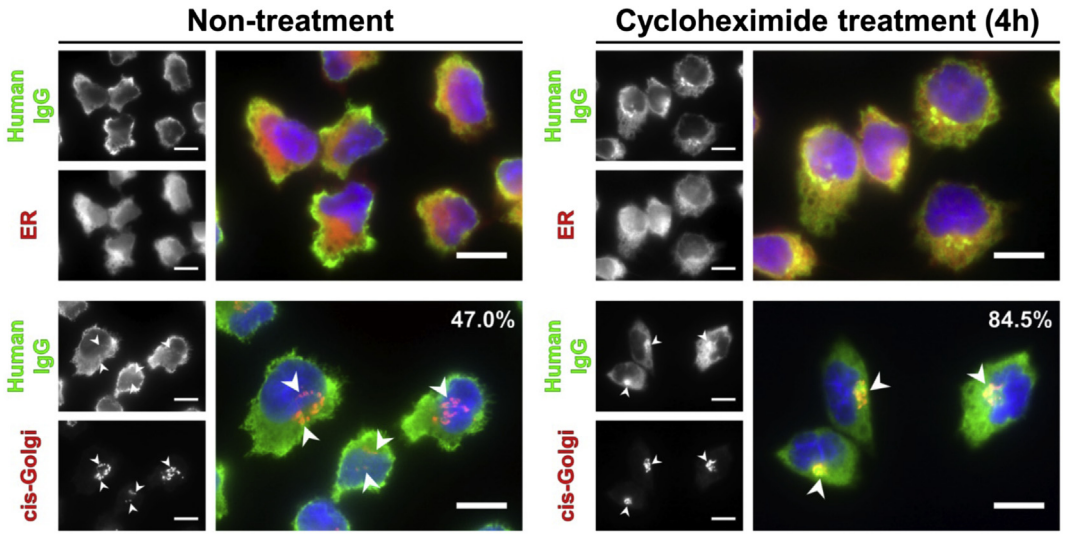

FIG. 4. Immunofluorescent microscopy of CHO-IgG3 cells. Cells were cultured with (right panels) or without (left panels) cycloheximide (CHX). Green indicates IgG3, red indicates the endoplasmic reticulum (ER) or cis-Golgi, and blue indicates nucleus. Many cells showed co-localization of IgG within the cis-Golgi, regardless of CHX treatment (lower panels, arrowheads). The ratios of cells which human IgG co-localized within cis-Golgi are shown in the merge images (bottom panels). Bars: 10 μm.

IgG secretion from CHO cells, based on the comparison of cells stably expressing different types of recombinant IgG. In the present study, the cultured CHO cells produced antibodies which $t_{1/2}$ of its HC of IgG1A, B, and IgG3 were >4 h, >8 h, and >2 h, respectively, and that of the LC were >2 h, >4 h, and >4 h, respectively. The secretion of recombinant IgG by stably expressing CHO cells was slower compared with plasmacytoma cells but faster than transiently transfected cells. Thus, the secretion of IgG from CHO cells can be improved.

By chase assay, all types of IgG could not be secreted completely, even after the amount of IgG polypeptides in the cells were decreased by CHX treatment. In other words, the overloading of nascent polypeptides into the ER is not the only reason for the limited IgG secretion—a constant amount of synthesized polypeptide cannot be secreted.

To determine why the remaining IgG could not be secreted, immunofluorescent microscopy and SEC were performed. After the incubation of cells with CHX, the reduction of green fluorescent intensity was observed by fluorescent microscopy because intracellular IgG were secreted to the medium. It seems to be good idea to discuss the amount of retained IgG in comparison with the results of chase assay and immunofluorescent microscopy, but it was difficult to discuss the retention from green fluorescent intensity because the shape and size of cells varied. Investigation of the changes in green fluorescent intensity by quantitative analysis technique such as flow cytometry might lead to an accurate

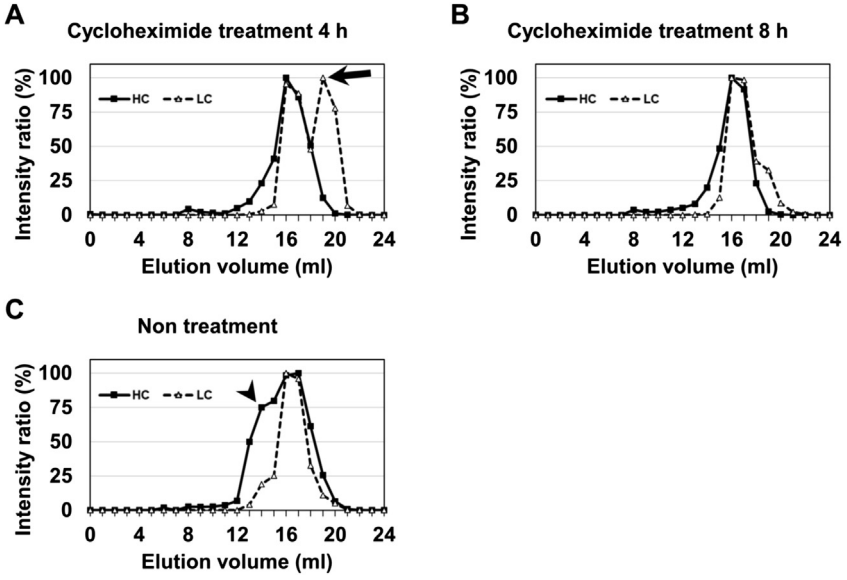

FIG. 5. Size exclusion chromatography (SEC) of intracellular IgG1A. CHO-IgG1A cells were cultured with cycloheximide (CHX) for 4 (A) or 8 h (B), and cell lysate proteins were separated by SEC. IgG1A in separated protein fractions was detected by western blot. Changes in immunoglobulin heavy (HC) and light (LC) chain band intensities are shown in graphs, with the peak intensity set to 100% for HC and LC. Proteins in control cells that were cultured in the absence of CHX were also analyzed (C). Significant peaks of LC (A, arrow) and aggregate or oligomer IgG (C, arrowhead) were seen.

discussion. This will be the next task for the further detailed understanding of IgG retention in the future. 4 h after the inhibition of protein synthesis, intracellular IgG was retained in all cell lines. Nascent polypeptides of IgG are first folded and modified in the ER and then transferred to the Golgi apparatus. As shown in Figs. 2–4, IgG co-localization within the ER was observed in all cell lines, regardless of CHX treatment. Consequently, IgG accumulated primarily in the ER, suggesting that the bottleneck of IgG secretion occurs mainly in the ER.

The ratio of cells showing partial co-localization of IgG with the cis-Golgi was measured to evaluate the progress of intracellular IgG secretion (Figs. 2–4). A recent fluorescent microscopy study showed that difficult-to-express antibodies hardly co-localize within the cis-Golgi, in contrast to classical IgG1 (39). The ratio of cells showing co-localization of IgG within the cis-Golgi in CHO-IgG1B cells (17.3% and 11.0% before and after CHX treatment, respectively) was smaller than in the other two cell lines (52.0% and 24.2% before and after CHX treatment in CHO-IgG1A cells and 47.0% and 84.5% before and after CHX treatment in CHO-IgG3 cells). This result may reflect the serious imbalance in HC and LC polypeptides in CHO-IgG1B cells (Fig. 1D). As LC and HC are transported from the ER to the Golgi apparatus after assembly into IgG, a shortage of LC peptides might result in lower co-localization of IgG within the cis-Golgi. We do not know why the ratio of CHO-IgG3 cells showing co-localization of IgG with the cis-Golgi did not decrease after CHX treatment. However, these parameters may be useful to evaluate the efficiency of secretion. No crystals (40,41) or aggregates (35) of IgG were observed in any cell lines (Figs. 2–4).

To explain the retention of IgG in the ER, IgG1A maturation was investigated by SEC after CHX treatment. The amount of aggregate or oligomer IgG disappeared after protein synthesis was inhibited and might have been secreted or degraded during the incubation. The LC peak that was seen 4 h after the inhibition cannot be explained. Even 8 h after the inhibition of protein synthesis, most of the IgG appeared to be fully assembled, meaning that folding and disulfide bonding had been completed. Taking these results into account, the remaining IgG may lack the proper glycosylation or might encounter problems in the protein quality control steps in the ER after the complete assembly.

One explanation for the incomplete IgG secretion, despite intracellular HC or LC polypeptide levels of more than 20%, is an impaired UPR in the ER. In contrast to previous studies that used pulse-chase assays with radioisotopes, the current chase assay using CHX decreased the amount of newly synthesized proteins in the ER, which might have weakened the UPR and led to inefficient translation. Translation, post-translational modifications, and transportation of proteins must be orchestrated to achieve efficient secretion. Examining proteins that are involved in the UPR by chase assay may be necessary to fully explain the intracellular retention of IgG.

Another explanation for the incomplete secretion of IgG is the intracellular imbalance in HC and LC polypeptides. It has been repeatedly demonstrated that the LC:HC ratio at the mRNA and polypeptide levels is important in determining the cellular production of IgG (2,16). Some studies have demonstrated that there is no correlation between LC and HC mRNA levels and antibody production at higher mRNA levels (2,16,42,43), whereas other studies have shown that antibody production is related to LC and HC mRNA levels (32,44,45). Maximum IgG production could be achieved by co-expression of an equal molar HC and LC vectors (46). On the other hand, an excess of intracellular LC polypeptide resulted in high IgG production, even though the gene copy number of HC is higher than that of LC (47). Another study found that a higher LC:HC gene copy number ratio was required to improve the production of a difficult-to-express IgG (31).

Although the ratio of HC and LC mRNA levels was not measured in the current study, the HC and LC polypeptides had different rates of intracellular decline. In the CHO-IgG1A and B cells, the LC polypeptide content decreased faster than the HC polypeptide; CHO-IgG1B cells retained >90% of HC but only 40% of LC. The CHO-IgG3 cells experienced a faster reduction in HC polypeptide compared to that of LC. Excess LC might result in efficient transportation of IgG to the Golgi apparatus because the higher percentage of CHO-IgG3 cells showed co-localization of IgG within the cis-Golgi. The times for HC and LC polypeptides to reach stable intracellular amounts and for the amount of secreted IgG to plateau appear to be linked. Neither HC nor LC completely disappeared in all cell lines, but overloading of the HC or LC polypeptide may be necessary to secrete all fully assembled IgG.

Here, we showed that more than 20% of synthesized recombinant IgG could not be secreted from CHO cells. The bottleneck of IgG secretion appeared to occur in the modification or transportation of fully assembled IgG in the ER. In addition to classical cell engineering techniques, such as the overexpression of proteins that are related to the protein secretory pathway or UPR and the optimization of the HC:LC ratio at the mRNA and polypeptide levels, improvements to further modifications after the formation of fully assembled IgG, such as glycosylation, might enhance production through the efficient usage of HC and LC in the ER. Conversely, cell line-specific engineering that is based on quantitative analyses will be necessary for the efficient establishment of high-producer cells.

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