Analysis of the immunoglobulin G (IgG) secretion efficiency in recombinant Chinese hamster ovary (CHO) cells by using Citrine-fusion IgG

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Abstract Biopharmaceuticals represented by immunoglobulin G (IgG) are produced by the cultivation of recombinant animal cells, especially Chinese hamster ovary (CHO) cells. It is thought that the intracellular secretion process of IgG is a bottleneck in the production of biopharmaceuticals. Many studies on the regulation of endogenous secretory protein expression levels have shown improved productivity. However, these strategies have not universally improved the productivity of various proteins. A more rational and efficient establishment of high producer cells is required based on an understanding of the secretory processes in IgG producing CHO cells. In this study, a CHO cell line producing humanized IgG1, which was genetically fused with fluorescent proteins, was established to directly analyze intracellular secretion. The relationship between the amount

of intracellular and secreted IgG was analyzed at the single cell level by an automated single-cell analysis and isolation system equipped with dual color fluorescent filters. The amounts of intracellular and secreted IgG showed a weak positive correlation. The amount of secreted IgG analyzed by the system showed a weak negative linear correlation with the specific growth of isolated clones. An immunofluorescent microscopy study showed that the established clones could be used to analyze the intracellular secretion bottleneck. This is the first study to report the use of fluorescent protein fusion IgG as a tool to analyze the secretion of recombinant CHO cells.

Keywords Chinese hamster ovary cell · Therapeutic antibody production · Single clone analysis and isolation · Fluorescent protein fusion antibody · Yellow fluorescent protein (YFP) · Intracellular IgG secretion

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Introduction

Several types of recombinant immunoglobulin G (IgG) used as biopharmaceuticals are produced by the cultivation of recombinant mammalian cells, especially Chinese hamster ovary (CHO) cells. Considering the growing demand for antibody drugs (Ecker et al. 2015), more efficient and lower-cost

production of IgGs is required. Both cell engineering and cell culture engineering strategies have improved productivity, resulting in very high titers (Wurm 2004; Li et al. 2010; Lai et al. 2013; Bandaranayake and Almo 2014; Takagi et al. 2017; Kuwae et al. 2018). However, although high titers were achieved through long-term selection and repeating the optimization of culture processes, it is still challenging to establish high producer cells.

In previous studies, the specific productivity of recombinant CHO cells was improved by modifying each step in the protein synthesis machinery, such as the amplification of genes encoding the target proteins, (Omasa 2002; Kawabe et al. 2012), gene optimization for efficient transcription (Hung et al. 2010), and the overexpression of secretory proteins for efficient post-translational modifications and transportation of proteins to the extracellular region (Baek et al. 2015; Zhou et al. 2018). The intracellular protein secretion processes including folding, assembly, modifications, and transportation of proteins in cells have been the focus of research, because it is thought that the secretion of antibodies is a bottleneck during production. This was suggested because higher mRNA levels do not correlate with increased protein synthesis (Dinnis and James 2005; Barnes and Dickson 2006). Many studies have tried to improve the bottleneck during the intracellular secretion of target proteins by the regulation of endogenous secretory proteins, such as those which perform protein modification in the endoplasmic reticulum (ER), (Borth et al. 2005). An improvement in the fusion efficiency of secretory vesicles increased productivity (Peng and Fussenegger 2009; Peng et al. 2010; Peng et al. 2011). The improvement of protein secretion by the overexpression of transcription factors involved in the unfolded protein response was also effective at increasing productivity (Tigges and Fussenegger 2006; Ohya et al. 2008; Nishimiya et al. 2013).

However, the effect of these strategies is restrictive and has not always improved productivity depending on the proteins and cells involved (Davis et al. 2000; Peng et al. 2010; Pybus et al. 2014). This may be because of the complicated secretory and signaling pathways in recombinant highly-productive CHO cells, which might be different from that in natural cells. Even though the intracellular secretion process is thought to be a bottleneck for IgG production, there have been few analyses and discussions regarding IgG

secretion in recombinant CHO cells cultured in suspension without serum. To achieve a more efficient and rational process design for the establishment of high producer cells, it is necessary to build a platform to improve the protein secretion process based on the fundamental analysis of secretory pathways in recombinant cells producing IgG.

In this study, we established a CHO cell line producing IgG1 fused with Citrine, a modified yellow fluorescent protein with a molecular weight of 27 kDa, as a tool for the direct analysis of intracellular IgG secretion. Using the conventional and direct labeling of IgG, the relationship between the amount of intracellular and secreted IgG was analyzed at the single cell level by a single cell isolation system equipped with dual color filters. The characteristics of the isolated clones were analyzed to investigate the efficient secretion of synthesized IgG in the cells. The use of the Citrine-fusion IgG (IgG-Citrine) for the analysis of intracellular secretion was also investigated by fluorescent microscopic observation of the cells to determine the bottleneck in IgG secretion.

Materials and methods

Construction of the IgG-Citrine expression vector

The expression vector of IgG1 whose light chains (LC) are fused with Citrine at the C-terminus via a $(G_4S)_2$ flexible linker (Fig. 1a, Haas et al. 2010) was constructed as follows. The sequence of IgG1 LC in the pUC57 vector, a kind gift from Dr. Onitsuka in Tokushima University, was cut out by polymerase chain reaction (PCR) using KOD Plus Neo (Toyobo, Osaka, Japan) and the following primer sets to insert the $(G_4S)_2$ linker: Forward: GCC GCC ACC ATG GTG CTG CAG, Reverse: TGA TCC TCC TCC TCC TGA TCC TCC TCC TCC GCA CTC GCC CCG GTT GAA G. The amplified LC sequence was inserted into the Citrine-N1 vector (Plasmid #54593, Addgene, Cambridge, MA, USA) using an In-Fusion HD Cloning Kit (Takara Bio, Kusatsu, Japan). The insertion sequence was prepared by PCR using the amplified LC sequence as a template and the following primer sets: Forward: CTC GAG CTC AAG CTT GCC GCC ACC ATG GTG CTG, Reverse: CTC GCC CTT GCT CAC TGA TCC TCC TCC TCC TGA. The Citrine-N1 vector was linearized by inverse PCR using

Fig. 1 **a** Structure of designed IgG1 fused with Citrine at the C-terminus of the light chain. **b** Schematic diagram of the detection of intracellular and secreted IgG by dual color fluorescence in an automated single-cell analysis and isolation system. A single cell was trapped in a single well. IgG-Citrine producing cells were detected in the green fluorescence channel (FITC) because Citrine was fused with the LC. The secreted IgG was detected in the deep red fluorescence channel (APC)

because it was detected by anti-human IgG tagged with Cy5 by forming a sandwich with anti-human IgG Fc and the target IgG. **c** Example images of IgG-Citrine producing cell clone 1 trapped in a well. Bright field (left), FITC (middle), and APC (right) images were obtained for each cell. The cell emitted green fluorescence under the FITC channel (middle) and the edge of the well emitted red fluorescence (right) under the APC channel

the following primer sets to remove multi-cloning site sequences and the first ATG start codon of the Citrine gene: Forward: GTG AGC AAG GGC GAG GAG CT, Reverse: AAG CTT GAG CTC GAG ATC TG. The constructed Citrine-fusion LC sequence was ligated into pELX2.2 Mammalian PowerExpress System (Toyobo) by Mighty Mix (Takara Bio) after digestion with *HindIII* (Takara Bio) and *NotI* (Takara Bio).

The sequence of the IgG1 heavy chain (HC) in the pUC57 vector, a kind gift from Dr. Onitsuka in Tokushima University, was cut out by PCR using the following primer sets: Forward: AAG CTT CGT ACG CCC GCC GCC ACC ATG GGC TCC, Reverse: CGC CTG ACG CGT CCC TCA CTT CCC GGG GCT CAG GCT C. By using the In-Fusion HD Cloning Kit, the amplified HC sequence was inserted into the pEHX1.1 Mammalian PowerExpress System

(Toyobo) which was linearized by *SmaI* (Takara Bio) in advance.

Both the pEHX1.1 and pELX2.2 vectors containing the HC and Citrine-fusion LC sequences, respectively, were digested by *BglII* (Takara Bio) and *EcoRI* (Takara Bio), ligated by Mighty Mix and termed the pEHCLC vector. Endotoxin was removed from the constructed pEHCLC vector by the MiraCLEAN Endotoxin Removal Kit (Takara Bio).

Establishment of IgG-Citrine producing cells

CHO-K1 adherent cells were maintained in Iscove's modified Dulbecco's medium (IMDM, Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS, Sigma Aldrich). The culture medium was replaced with BalanCD Transfectory CHO (Irvine Scientific, Santa Ana, CA, USA) for 8 h before

transfection, and the cells were transfected with 1.2 µg of pEHCLC vector linearized with *SspI* (Takara Bio) after mixing with polyethyleneimine. Twenty-two hours later, the culture medium was replaced with IMDM containing 10% FBS and selected by 15 µg/ml of puromycin (InvivoGen, San Diego, CA, USA). The transfected cells were adapted to the suspension culture by gradually replacing the medium to BalanCD CHO GrowthA medium (Irvine Scientific) with 6 mM L-glutamine (FUJIFILM Wako Pure Chemical, Osaka, Japan) and gradually reducing the FBS concentration in the presence of puromycin. The adapted cell pool was maintained in a 125 ml Erlenmeyer flask at 37 °C, 80% humidity, 5% CO₂, and 90 rpm on a Climo-Shaker ISF1-XC (Kuhner, Birsfelden, Switzerland) or in a 10-cm dish at 37 °C with 5% CO₂.

Single clone isolation by automated single-cell analysis and isolation system

An automated single-cell analysis and isolation system (Yoshimoto et al. 2013; Yoshimoto and Kuroda 2014, AS ONE, Osaka, Japan) was used to analyze the relationship between the amount of IgG in cells and secreted into the medium. An amino-coated micro chamber with ø30 µm wells was purchased from AS ONE and coated with 20 µg/ml of goat anti-human IgG-Fc fragment antibody (A80-104A, Bethyl Laboratories, Montgomery, TX, USA) at 4 °C, overnight. Sodium azide was removed from the antibody solution in advance by replacing the solution with phosphate buffered saline (PBS, 137 mM NaCl, 8.10 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄, pH 7.4) using a Microcon centrifugal 30 K filter device (Merck, Darmstadt, Germany). The cells producing IgG-Citrine with a molecular weight of 200 kDa were maintained in a 10-cm dish with medium containing 1% FBS. After washing the chamber with PBS 3 times, these cells suspended in a medium were applied to the chamber and incubated at 4 °C for 10 min to allow them to fall into the wells. After gently washing the chamber with medium to remove floating cells, the cells were incubated with the medium containing 1% FBS at 37 °C for 1 h to allow them to secrete IgG-Citrine. Then, 2 µg/ml of goat anti-human IgG H&L Cy5 conjugate (ab97172, Abcam, Cambridge, UK) was applied simultaneously to detect secreted IgG-Citrine (Fig. 1b). After washing the chamber with

medium, the area intensities of fluorescein isothiocyanate (FITC, excitation: 465–495 nm, emission: 515–555 nm) and allophycocyanin (APC, excitation: 628 nm, emission: 692 nm) in 15,870 wells were measured. The linear correlation between FITC and APC fluorescent intensities was investigated by Spearman's rank correlation analysis using R software (<https://www.r-project.org/>). Ninety-six clones with different fluorescent intensities were isolated to 384 well plates with 50 µl of the medium containing 1% FBS and incubated at 37 °C and 5% CO₂. The cells were cultured with 1% FBS during this isolation step because the isolated cells did not grow well in the serum-free medium, which might be caused from a shortage of growth factors. Notably, the cells did not attach to the bottom of the 384 well plates even after the addition of 1% FBS. After the cell population recovered in the 384 well plates from a single cell, the cell culture was scaled up to a 125 ml Erlenmeyer flask with 30 ml of medium and maintained at 37 °C, 80% humidity, 5% CO₂, and 90 rpm. FBS was eliminated during this step.

Purification of IgG-Citrine from culture medium

IgG-Citrine was purified based on the methods of Haas et al. (2010) using a Protein A column and size exclusion chromatography (SEC). This purified IgG-Citrine was used as a standard for the enzyme linked immunosorbent assay (ELISA) described below. IgG-Citrine was collected by applying filtered culture supernatant of CHO cells producing IgG-Citrine onto a HiTrap rProtein A FF 1 ml column (GE Healthcare, Uppsala, Sweden), which was equilibrated with PBS in advance. After washing out the remaining proteins with PBS, IgG-Citrine was eluted with 0.1 M citrate buffer (mixture of 0.1 M citric acid and 0.1 M trisodium citrate dihydrate, pH 2.8) and the eluent was immediately neutralized by 1 M Tris-HCl (pH 9.0). The buffer was replaced with PBS by an Amicon Ultra 0.5 ml 10 K filter column (Merck). Concentrated proteins were loaded onto an ÄKTA Prime Plus equipped with a SuperoseTM 6 10/300 GL column (GE Healthcare). The fractions containing IgG-Citrine monomers were collected and concentrated by Amicon filter column. The protein concentration was determined by absorbance at 280 nm based on the amino acid sequence.

Growth and productivity analysis

To evaluate the growth and productivity of the isolated single clones, 12 clones with different fluorescent intensities were analyzed in batch culture conditions (in 125 ml Erlenmeyer flasks with 30 ml medium containing 6 mM L-glutamine at 37 °C, 80% humidity, 5% CO₂, and 90 rpm) started by seeding the cells at 5×10^5 cells/ml on day 0. The cultivation was continued until the cell viability decreased to less than 70%. One ml of culture was sampled every day, and 500 µl was used to analyze the viable/total cell density and mean diameter of the viable cells by Vi-Cell XR (Beckman Coulter, Brea, CA, USA). Cell volume during the cultivation was calculated from the mean diameter of cells each day using the following equation, hypothesizing the cell as a sphere:

$$V = \frac{4}{3} \pi \left(\frac{d}{2} \right)^3$$

where V = cell volume (fL) and d = mean cell diameter (µm). Another 500 µl was centrifuged at $17,000 \times g$ for 2 min, and 400 µl of the supernatant was frozen at -80 °C until use for ELISA. ELISA was performed based on the methods described in previous reports (Kim et al. 2010; Onitsuka et al. 2012). Briefly, 96-well EIA/RIA plates (Corning, Corning, NY, USA) were coated with 100 µl of 2 µg/ml goat anti-human IgG-Fc fragment antibody (A80-104A, Bethyl Laboratories) diluted with KPL coating solution (SeraCare Life Sciences, Milford, MA, USA) and incubated at room temperature (RT) for more than 1 h. Then, the plate was blocked with 300 µl of 1% (w/v) bovine serum albumin (BSA)/PBS at RT for more than 1 h. After washing the wells with wash buffer (154 mM NaCl and 0.05% Tween 20), the reference IgG-Citrine and culture supernatant samples diluted with 1% BSA/PBS were applied to the plate in triplicate at 100 µl per well. After incubation at 37 °C for more than 1 h, the plate was washed with wash buffer. Then, 100 µl of 0.286 µg/ml anti-human IgG-Fc fragment antibody HRP conjugate (A80-104P, Bethyl Laboratories) diluted with 1% BSA/PBS was applied to each well and incubated at 37 °C for 30 min. After washing with wash buffer, 100 µl of KPL ABTS Peroxidase substrate solution mix (SeraCare Life Sciences) was applied and the reaction was stopped by 100 µl of KPL ABTS Peroxidase Stop Solution (SeraCare Life

Sciences). The absorbance at 405 nm was measured by an EnSpire 2300 Multilabel Reader (PerkinElmer, Waltham, MA, USA) and the concentration of IgG-Citrine in each culture supernatant sample was calculated from a standard curve approximated with 4 parameters. The specific growth and specific productivity were calculated from the concentration of viable cells and IgG-Citrine depending on the time course (Omasa et al. 1992). Linear correlations between the 5 parameters (APC area intensity, FITC area intensity, specific growth, specific productivity, and cell volume) were investigated by Spearman's rank correlation analysis using R software.

Immunofluorescent microscopy

The preparation of samples for fluorescent microscopy observation was performed as previously described (Kaneyoshi et al. 2018). In brief, CHO cells producing IgG-Citrine maintained in 6-well plates were passaged into 12-well plates with 1 ml IMDM (10% FBS) and poly-L-lysine coated coverslips at 37 °C and 5% CO₂ for 2 nights. To stop nascent peptide synthesis, the medium was changed to serum-free IMDM with 50 µg/ml cycloheximide (CHX) and incubated at 37 °C, 5% CO₂ for 4 h. After washing with PBS, the cells were fixed in 4% paraformaldehyde/PBS at RT for 15 min and were permeabilized with 0.1% Triton X-100/PBS for 5 min. The cells were blocked with 5% BSA/PBS at RT for 1 h and incubated with primary antibody (anti-GM-130 (35/GM130) mouse antibody (610822, BD Biosciences, San Jose, CA, USA)) diluted in 5% BSA/PBS (1:200) at 4 °C overnight. The next day, the cells were incubated with secondary antibodies (Goat anti-mouse IgG H&L Alexa Fluor® 647-conjugate (ab150119, Abcam)) diluted in 5% BSA/PBS (1:500) at RT for 1 h. The cells were then counterstained with 1 µg/ml of 4',6-diamidino-2-phenylidole (DAPI) for 5 min. Coverslips were fixed on the glass slides with Dako fluorescence mounting medium (DAKO North America, Carpinteria, CA, USA).

A BZ-X710 (Keyence, Osaka, Japan) equipped with DAPI, GFP, and Cy5 filters was used. Pseudocoloring and normalization of contrast were performed using ImageJ software (<https://imagej.nih.gov/ij/>).

Results

Analysis of IgG-Citrine producing cells by dual color fluorescent intensity

The pool of CHO cells producing IgG-Citrine was analyzed by an automated single-cell analysis and isolation system with a dual fluorescent filter set containing FITC and APC. It was expected that the intensity of FITC (green) would reflect the amount of intracellular IgG fused with Citrine, and that the intensity of APC (deep red) would reflect secreted IgG detected by anti-human IgG conjugated with Cy5. After the incubation of cells in the chamber for 1 h, a bright field image and dual color fluorescent images were obtained (Fig. 1c). Green fluorescence was observed in cells trapped in the wells, and deep red was observed around the edge of the wells. These three sets of images were obtained for 15,870 wells and the area intensity for each fluorescent channel was measured. Cells seemed to be present in 4012 wells with an FITC intensity of ≥ 125.81 (approximately 25%). Though the presence or absence of cells could not be judged only from FITC intensity accurately, the wells with FITC intensity below 125.81 were disregarded. The area intensity of FITC and APC in each well was plotted in Fig. 2. The area intensities of fluorescence showed a linear correlation with correlation coefficient and p -values of 0.506 and 2.2×10^{-16} , respectively.

Analysis of the growth and productivity of batch cultured clones

Twelve clones, indicated by red circles (Fig. 2), were chosen to be analyzed on the basis of their specific growth and productivity. These clones were different from the main group of analyzed cells and did not show a positive correlation between the FITC and APC intensities. The clones were numbered depending on the APC intensity as summarized in Table 1. The SEC analysis of IgG secreted from an isolated clone followed by the detection of human IgG and Citrine by western blotting showed that the cells secreted both the fully assembled IgG and free Citrine conjugated LC (data not shown). Before the analysis, IgG-Citrine was purified from the supernatant of the cell culture to be used as a reference in the ELISA study. IgG-Citrine was separated using a Protein A

Fig. 2 2D plot graph showing the correlation between the area intensity of FITC (horizontal axis) and APC (vertical axis) channels. The chambers, which showed an FITC intensity less than 125.81, were eliminated because these did not contain cells. The intensity of these two colors showed a weak linear correlation ($r = 0.506$). Some cell populations did not secrete any IgG-Citrine (red ellipse). Twelve clones indicated by red circles were named clones 1–12 in the order of APC area intensity. (Color figure online)

column; however, a subsequent SEC analysis showed that more than 60% of IgG-Citrine formed aggregates (data not shown). The concentrated monomer IgG-Citrine solution was bright yellow as previously reported (Haas et al. 2010).

The growth and changes in IgG-Citrine titers from each clone are summarized in Fig. 3. The cell growth in all clones was almost stopped by day 4. Clones with lower numbers (= higher APC intensity) tended to have slower growth compared with the other clones. Most clones showed an increase in cell volume in the early part of cultivation, which decreased thereafter (Fig. 4). Although the cell volume of all clones decreased to around 1000 fL by the end of cultivation, the maximum volume was variable in the 12 clones.

The specific growth and specific productivity of each clone were calculated based on the changes in cell density and IgG titer (Table 1). The cell volume on day 3 (Table 1) was used for the analysis below as a parameter indicating cell size in the exponential growth phase. The linear correlation coefficient (r) and p values among the 5 parameters (APC area

Table 1 APC area intensity, FITC area intensity, cell volume, specific growth, and specific productivity of 12 isolated clones

Clone	APC area intensity	FITC area intensity	Specific growth (1/h)	Specific productivity (pg/cell/day)	Day 3 cell volume (fL)
1	116.23	199.38	0.0208	3.21	2781
2	88.35	205.56	0.0270	0.643	3042
3	87.84	139.86	0.0258	0.875	1926
4	87.74	161.75	0.0304	1.05	2068
5	75.42	156.34	0.0240	0.848	2786
6	72.98	152.71	0.0326	4.08	3477
7	70.70	172.10	0.0394	0.525	3255
8	69.48	185.01	0.0240	0.536	3098
9	43.77	192.97	0.0309	1.07	1979
10	38.74	205.56	0.0276	1.06	2029
11	34.62	190.40	0.0338	0.788	2052
12	23.49	194.71	0.0322	1.20	2626

intensity, FITC area intensity, specific growth, specific productivity, and cell volume) were calculated by Spearman's rank correlation analysis (Fig. 5). The results showed that only the APC area intensity and specific growth was statistically correlated to each other at the 10% level. The APC area intensity showed a weak linear correlation with specific growth (Fig. 5a, $r = -0.571$, $p = 0.0525$), but not with specific productivity (Fig. 5b, $r = -0.00699$, $p = 0.991$) or cell volume (Fig. 5c, $r = 0.182$, $p = 0.573$). Furthermore, FITC area intensity showed no correlation with specific growth (Fig. 5d, $r = -0.0982$, $p = 0.761$), specific productivity (Fig. 5e, $r = 0.0666$, $p = 0.837$), or cell volume (Fig. 5f, $r = -0.119$, $p = 0.712$). The cell volume on day 3 showed no correlation with specific growth (Fig. 5g, $r = 0.0911$, $p = 0.778$) or specific productivity (Fig. 5h, $r = -0.203$, $p = 0.528$). There was also no correlation between specific growth and specific productivity (Fig. 5i, $r = -0.0315$, $p = 0.923$).

Fluorescent microscopy of fixed IgG-Citrine producing clones

We analyzed the localization of IgG-Citrine in all clones. Three representative clones with different secretory capacity are shown in Fig. 6. IgG-Citrine, which was distributed in the cells forming a complicated tubular network, was observed without immunofluorescent staining in all 12 cell lines as

shown by the representative 3 clones (Fig. 6, left panels). Immunofluorescent staining against GM-130, a cis-Golgi matrix protein with a molecular weight of 130 kDa, (Fig. 6, middle panels) showed IgG-Citrine co-localization within the cis-Golgi (Fig. 6, right panels, arrows). The co-localization of IgG-Citrine within the cis-Golgi was observed even at 4 h after the inhibition of nascent peptide synthesis by CHX despite a decrease in the intensity of green fluorescence from Citrine, suggesting that IgG-Citrine remained in the cis-Golgi for more than 4 h.

Discussion

This study investigated the relationship between IgG synthesized in cells and secreted into medium using a CHO cell line producing a fluorescent protein fusion IgG and a single-cell analysis and isolation system. Citrine, a modified yellow fluorescent protein, was used in this study because it is well expressed in the ER (Griesbeck et al. 2001). In addition, the titer of the antibodies fused with Citrine was higher than that of antibodies fused with representative fluorescent proteins (Haas et al. 2010). A (G₄S)₂ flexible linker was used to connect Citrine to C-terminus of IgG LC because this combination showed the highest titer in production by transiently transfected HEK293 cells (Haas et al. 2010). As expected, this conventional and direct labeling enabled us to detect IgG in living cells;

Fig. 3 Changes in total cell density (opened circles), viable cell density (closed circles), and IgG-Citrine titer (closed squares) were analyzed for each isolated clone. The numbers at the top left of the graphs indicate clone numbers

however, the cells partially secreted Citrine-fusion LC without assembly into an IgG molecule. It is known that cells producing recombinant IgG can secrete free LC into medium without assembling into a heterodimer IgG molecule (Bhoskar et al. 2013; Ishii et al. 2014). Thus, the leakage of Citrine-fusion LC without assembly is not uncommon. However another possible reason is inefficient folding and assembly of LC because of interference by the large Citrine tag (= 27 kDa). In addition, this large tag was probably why we observed more than 60% IgG-Citrine purified

by Protein A column formed aggregates, despite a previous report showing less than 10% aggregates (Haas et al. 2010). Even though Citrine is easily expressed in mammalian cells, it is not always effective for the analysis of IgG, which requires complicated post-translational modifications.

Though Citrine fusion IgG showed some demerits as written above, we found that the CHO cells producing IgG-Citrine is useful to analyze the secretion processes. The duration until the secretion of IgG reaches a plateau was analyzed by chase assay as

Fig. 4 Changes in the volume of cells measured for each clone. The numbers at the bottom left of the graphs indicate clone numbers. The cell volume on day 3 indicated by arrows was

used to analyze the relationship between the cell volume and fluorescent intensities, specific growth, and specific productivity

performed in our previous report (Kaneyoshi et al. 2018). Three clones isolated in this study were analyzed, and we found that the secretion of IgG was nearly completed by 6 h after the synthesis in the ER, and about 20% of the IgG retained in the cells (data not shown), similar to the cells producing the native therapeutic IgG; the secretion almost reached a plateau within 4–6 h and at least 20% of the IgG components retained in the cells (Kaneyoshi et al.

2018). As written above, fluorescent microscopy showed that the IgG-Citrine distributed in the cells formed tubular networks as shown in the cells producing native IgG (Hasegawa et al. 2011; Mathias et al. 2018). This IgG-Citrine was roughly overlapped with the ER (data not shown). In addition, we found that the LC fused with Citrine accurately co-localized with the HC as detected by immunofluorescent staining (data not shown), that the LC fused with

Fig. 5 The relationships between APC area intensity and specific growth (a), specific productivity (b), or day 3 cell volume (c) of the 12 clones were analyzed. As for graphs a–c, the relationship between each factor and FITC intensity was also analyzed (d–f). The relationship between cell volume and specific growth (g), or specific productivity (h), and between specific growth and specific productivity (i) were also analyzed.

Correlation coefficient (r) and p values are shown in the top right. The coefficient of determination (R^2) of the linear regression is shown in the bottom left. The specific growth and APC fluorescent intensity showed a weak negative correlation (a, $r = -0.571$), otherwise the other combinations showed no correlation (b–i).

Citrine can assemble with the HC and then be secreted. The secretion processes of IgG-Citrine, which were revealed by fluorescent microscopy, is comparable to that of the native IgG, except for the longer retention in the cis-Golgi (discussed below). Based on the chase assay and immunofluorescent microscopy results, we concluded that the CHO cell producing IgG-Citrine can imitate native IgG producing cells.

Fluorescent activated cell sorting (FACS) systems have been used to enrich high production cells by detecting target proteins or co-expressed marker proteins either maintained in the cells or secreted outside (Mattanovich and Borth 2006; Sleiman et al. 2008; Kida et al. 2013; Yoshimoto and Kuroda 2014; Priola et al.

2016). Currently, other techniques to isolate high producers by detecting secreted proteins with specific antibodies fixed to micro wells are emerging (Park et al. 2011; Yoshimoto et al. 2013; Yoshimoto and Kuroda 2014; Priola et al. 2016). The single cell analysis system allowed us to analyze secreted proteins from a single cell by using a micro chamber without anchoring detection antibodies to the cells. The advantage of this system was that it did not enrich the target cell population by manually setting gates containing desired cell characteristics but by directly isolating single cell clones with different characteristics shown by fluorescent intensities using a thin capillary in a one-step assay (Yoshimoto et al. 2013).

Fig. 6 Observation of isolated clones under fluorescent microscopy. Cells were treated with translation inhibitor cycloheximide (CHX) or were untreated and then underwent chemical fixation and immunofluorescent staining. Each color (pseudocolor) in the merged images indicates the following: green: IgG-Citrine, red: GM-130 (proteins localized in the cis-Golgi), and blue: nucleus. Bar = 10 μ m. (Color figure online)

The relationship between FITC and APC area intensity was analyzed by Spearman's rank correlation analysis because these intensities did not follow a normal distribution. The fluorescent intensities of FITC and APC showed a weak positive correlation ($r = 0.506$). As expected, the greater the amount of synthesized IgG, the greater the amount of IgG was secreted into the medium. However, some population of cells secreted very low levels of IgG (Fig. 2, ellipse

with dotted line), suggesting the necessity of cell screening based on the direct detection of secreted but not intracellular IgG. Twelve representative clones that differed from the main group were isolated to analyze their secretion capacity because these clones were thought to have different secretory capacity from the main population of cells. We analyzed these clones to investigate the efficient secretion of synthesized IgG. The highest titer was achieved by clone 1,

resulting in > 60 mg/L (Fig. 3-1). This is higher than that secreted from transiently transfected HEK293 cells in a previous study (Haas et al. 2010). This may be because of the isolation of single clones that stably express IgG-Citrine. There were some difficulties during the cultivation of the cells. The maximum viable cell density was less than 1×10^7 cells/ml for all the clones, and the isolated clones did not maintain high viability. This may be caused by toxicity related to the fluorescent proteins (Liu et al. 1999; Priola et al. 2016). In addition, cell debris from the dead cells was recognized as cells by Vi-Cell, resulting in the continued increase in viable cell number in the latter part of the cultivation (Fig. 3, clone 1 and 11). From these situations, the specific growth and productivity were calculated from data in the stage of the cultivation where linearity was seen between $\int(Xv/Xt)dt$ and $\ln(Xt)$, and $\int(Xv)dt$ and IgG concentration, respectively.

To investigate the relationship between the fluorescent intensities and cell characteristics, Spearman's rank correlation coefficient was calculated because the number of isolated clones was not sufficiently large. The specific productivity showed no correlation with FITC area intensity (Fig. 5e). This may be caused by the imbalance between intracellular HC and Citrine fusion LC. IgG is secreted to the medium after the assembly of two HCs and LCs into a hetero-dimer molecule. Because Citrine was only conjugated with the LC, the FITC intensity did not reflect the amount of fully-assembled IgG, resulting in no correlation between FITC area intensity and specific productivity. The cell volume increased at the start of the cultivation and gradually decreased (Fig. 4). Similar changes in cell volume were reported in a mouse–mouse hybridoma (Frame and Hu 1990) and mouse myeloma NS0 cells (Khoo and Al-Rubeai 2009), which produced antibodies and were cultured in media containing serum. Non-transfected CHO-DUKX (Seewöster and Lehmann 1997) and antibody producing CHO cells derived from CHO-K1 cells (Martínez et al. 2015) cultured in serum free media also showed similar changes in cell size. In the current study, the FITC intensity did not show a significant correlation with the day 3 cell volume (Fig. 5f), although FITC reflects the amount of intracellular LC. Factors other than LC amount might determine the cell volume. It has been consistently reported that protein productivity and cell size are positively correlated (Lloyd et al.

2000; Kim et al. 2001). Another study showed that the repeated isolation of cells with the smallest forward and side scattering by FACS analysis was effective for obtaining high producer cells (Okumura et al. 2015). In the current study, the cell volume and specific productivity did not show a significant correlation (Fig. 5h). It is expected that the larger cells hold comparably more developed intracellular organelles which leads to a higher secretion capacity. Then, the low production level from large sized cells might not be caused from the lower secretion capacity, but by other reasons, such as less IgG copy number, less transcription efficiencies, etc. Further detailed investigation of such large cells with low secretion level from various points of views might give important clues to improve the production level.

As indicated by no correlation between the fluorescent intensities and the specific productivity (Fig. 5b and e), it is difficult to obtain high producer cells by using the fluorescent intensity alone, although this was achieved by previous FACS studies (Meng et al. 2000; DeMaria et al. 2007; Sleiman et al. 2008; Chuang et al. 2014). The reason for this discrepancy might be explained by the isolation of single cells without synchronization of the cell cycle. It is known that protein production is linked to the cell cycle (Lloyd et al. 2000; Yokota and Tanji 2008). Another explanation may be the instability of cellular protein production after isolation. It was reported that the ratio of GFP positive cells decreased after sorting by FACS (Zeyda et al. 1999). The automated single-cell analysis and isolation system, that we used in this study, was used to select IgG producing hybridoma cells in combination with cell surface fluorescence linked immunosorbent assay (Yoshimoto et al. 2013; Yoshimoto and Kuroda 2014). The cells with the highest levels of fluorescence were isolated, and quantification of secreted IgG by ELISA after 21 days of culture revealed that the isolated clones secreted comparably higher amounts of IgG than the parental cells. However, only 1 of 7 clones maintained very high secretion level without any decrease. Similar to this study, we performed ELISA more than 1 month after isolation, and the productivity might have changed during the maintenance cultivation, until the cell viability recovered. If the APC and FITC fluorescent intensities were measured again after expansion of culture scale, correlations between the fluorescent intensities and cell characteristics might be observed.

In order to establish clones stably secreting IgG, isolation after synchronization of cell cycle and repeated isolation of clones may be necessary. Therefore, it is challenging to obtain high producer cells that stably produce proteins over the long term.

Intracellular secretion was directly analyzed in all clones by fluorescent microscopy, and three representative images of clones are shown in Fig. 6. Again, the distribution of IgG in the network confirms previous reports (Hasegawa et al. 2011; Mathias et al. 2018). The co-localization of IgG-Citrine within the cis-Golgi was analyzed because a recent study showed that classical IgG1 co-localized within the cis-Golgi, despite a difficult-to-express bispecific antibody co-localized within the ER but not within the cis-Golgi (Mathias et al. 2018). All 12 clones showed IgG-Citrine partially co-localized within the cis-Golgi at 4 h after halting nascent peptide synthesis by CHX, indicating that IgG-Citrine remained in the cis-Golgi at least for 4 h, even though the overloading of nascent IgG from the ER was stopped. This long-term co-localization of IgG in the cis-Golgi was also observed in IgG3 producing cells in a previous report (Kaneyoshi et al. 2018). The bottleneck of IgG-Citrine secretion may be in the Golgi apparatus. The retention of IgG in the intracellular organelles has been discussed to construct mathematical models of IgG secretion. For example, Bibila and Flickinger (1991) showed a model using $t_{1/2G}$ (time required for 50% of IgG to be transported from Golgi apparatus to extracellular medium) = 20 min. Also, O'Callaghan et al. (2010) reported that the $t_{1/2G}$ by medium-high producer CHO cells stably expressing a recombinant chimeric IgG4 varied in a range from 0.09 to 1.59 h. Though the retention time seemed to be varied depending on the product and/or cell lines, at least 4-h retention of IgG within the cis-Golgi may be longer than usual. As many cells showed such long retention in the isolated clones, IgG-Citrine may require a longer time for modification in the Golgi apparatus compared with the native IgG because of its large tags. Though we have not analyzed the accurate retention time, it may be a good idea to link the results from the image analysis and IgG retention time investigated by chase assay using radio isotopes to construct a mathematical model and understand the factors which cause the bottlenecks in the secretion.

In conclusion, the relationship between intracellular and secreted IgG was analyzed by a newly

established CHO cell line producing IgG-Citrine. We found a weak correlation between intracellular and secreted IgG. However, it was very difficult to obtain clones with high specific productivity by single isolation dependent upon fluorescent intensities that reflect intracellular and secreted IgG, which might be caused by cell instability and heterogeneity. Further studies of these clones by quantitative analysis of IgG secretion dynamics and kinetics using time-lapse imaging may help identify targets that can be used to improve productivity.

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Compliance with ethical standards

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

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