EGCG improves recombinant protein productivity in Chinese hamster ovary cell cultures via cell proliferation control

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Abstract Chinese hamster ovary cell lines are good manufacturing practice-certified host cells and are widely used in the field of biotechnology to produce therapeutic antibodies. Recombinant protein productivity in cells is strongly associated with cell growth. To control cell proliferation, many approaches have previously been tested including: genetic engineering, chemical additives such as cell cycle inhibitors, and temperature shift of the culture. To be widely adopted in the biopharmaceutical industry, the culture methods should be simple, uniform and safe. To this end, we examined the use a natural compound to improve the production capacity. In this study, we focused on the antioxidants, catechin polyphenols, which are found in green tea, for cell proliferation control strategies. (–)-Epigallocatechin-3-gallate (EGCG), the major catechin that induces G0/G1 cell cycle arrest, was investigated for its effect on recombinant protein production. Adding EGCG to the cell culture media resulted in slower cellular growth and longer cell longevity, which improved the specific productivity and total yield of recombinant IgG1 in batch cultures

by almost 50% for an extra 2 or 3 days of culture. A lower L-glutamine consumption rate was observed in cells cultured in EGCG-containing media, which may be suggesting that there was less stress in the culture environment. Additionally, EGCG did not affect the N-glycan quality of IgG1. Our results indicated that adding EGCG only on the first day of the culture enhanced the specific productivity and total amount of recombinant protein production in batch cultures. This approach may prove to be useful for biopharmaceutical production.

Keywords Chinese hamster ovary cells · (–)-Epigallocatechin-3-gallate · Natural compound · G0/G1 phase arrest · Cell longevity · Recombinant protein production

Introduction

Chinese hamster ovary (CHO) cells account for the largest number of approved host cells used in manufacturing of biopharmaceuticals between 1982 and 2014, and during the most recently examined period from 2010 to July 2014 (Walsh 2014). Compared with other host cells such as *Escherichia coli* and *Saccharomyces cerevisiae*, CHO cells have an advantage in terms of post-translational modifications of recombinant proteins. Recombinant protein productivity in

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CHO cells is highly associated with cell growth. Many attempts have been undertaken to control cell growth (Kumar et al. 2007), such as cell engineering (Bi et al. 2004; Fussenegger et al. 1998; Strotbek et al. 2013), cell cycle inhibitors (Du et al. 2015), amino acid depletion (Fomina-Yadlin et al. 2014) and temperature shift to reduce the temperature of the cell culture (Kaufmann et al. 1999; Moore et al. 1997; Trummer et al. 2006). Several genes related to ribosome biogenesis and protein translation are highly expressed in the G1 phase of the cell cycle (Kumar et al. 2007). Cell cycle arrest, particularly in the G1 phase, has been used to enhance productivity by prolonging the production phase, in a number of commercially relevant cell lines such as hybridomas and CHO cells (Kumar et al. 2007). Although regulation of the cell cycle improves production in CHO cells, many of the methods take time and effort, have variable process set points, or have other issues (Du et al. 2015; Kumar et al. 2007). In this study, we attempted to use a natural compound found in food, to improve the production capacity with a simple and uniform method.

Oxidative stress causes several diseases (Ishizawa et al. 2014; Tajima et al. 2012; Yamano et al. 2015). Catechin polyphenols found in green tea (Tsujimura 1930, 1934) have health benefits due to antioxidant activity (Higdon and Frei 2003; Williamson and Manach 2005). Among them, (–)-epigallocatechin-3-gallate (EGCG) is the major catechin in green tea, and it has been suggested to be responsible for the majority of the potential health benefits attributed to green tea consumption (Nagle et al. 2006). Several studies have reported that EGCG induced G0/G1 cell cycle arrest (Ahmad et al. 2000a; Bae et al. 2009; Gupta et al. 2003; Ma et al. 2014). EGCG has been reported to have great potential as a cancer chemopreventive agent in clinical applications similarly to cell cycle modulators because of its safety, low cost and bioavailability (Singh et al. 2002, 2011). In normal cells, EGCG affects cell proliferation differently from carcinoma cells through different modulation of the transcription factor, nuclear factor- κ B (NF- κ B) (Ahmad et al. 2000b), or the activity of extracellular signal-regulated kinase (Erk) and protein kinase B (PKB)/Akt, and the expression of B cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) (Chung et al. 2003). In non-cancer cells, EGCG reversibly regulates the cell cycle-related genes, cyclin D1, cyclin E2, cyclin-dependent kinase 6 (Cdk6) and Cdk2

(Bae et al. 2009). These cell cycle-related genes are suppressed when cells are in a medium containing EGCG, but restored to the original levels after its removal (Bae et al. 2009). EGCG is not believed to have significant harmful effects on non-cancerous animal cells. For example, EGCG increases neuronal survival in vivo and in vitro (Ortiz-Lopez et al. 2016; Singh et al. 2016) and has anti-inflammatory properties in colitis models (Oz et al. 2013). In addition, EGCG is a water-soluble molecule, which means that it does not require extra chemicals for dissolution in the cell culture medium.

In this study, we report the effect of the tea polyphenol, EGCG, on cell proliferation control under several conditions and its effect on recombinant monoclonal antibody production. The effects of EGCG on the specific cell growth rate, specific recombinant protein production rate, final product concentration, cell metabolism and product quality of immunoglobulin G1 (IgG1) assessed by the N-linked glycosylation pattern were analyzed and discussed.

Materials and methods

Cell culture

A recombinant CHO-K1 cell line expressing the IgG1 antibody (Onitsuka and Omasa 2015) was used to evaluate the EGCG effect on antibody productivity. Cells were maintained in the custom-made serum-free medium, Top2 (Irvine Science, Santa Ana, CA, USA), containing 6 mM L-glutamine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 15 μ g/mL puromycin (InvivoGen, San Diego, CA, USA). Cells were cultured in 125-mL Erlenmeyer flasks (Corning Inc., Corning, NY, USA) incubated with shaking at 80 rpm, 37 °C, 5% CO₂ and 80% humidity using orbital Climo Shaker ISF1-X (Kuhner Shaker, Inc., Basel, Switzerland). An automated cell analyzer, Vi-cell XR (Beckman Coulter, Inc., Brea, CA, USA), was used to analyze total/viable cell concentrations.

Additive agent

EGCG (E4143, Sigma-Aldrich, St. Louis, MO, USA) dissolved in sterilized H₂O was used. Sterilized H₂O was used as vehicle control.

Cell culture for evaluation of productivity

The IgG1-expressing CHO-K1 cell line was seeded at 5×10^5 cells/mL in 125-mL Erlenmeyer flasks (Corning Inc.) with the serum-free medium Top2 (Irvine Science), containing 8 mM L-glutamine (Wako Pure Chemical Industries, Ltd.) and 15 μ g/mL puromycin (InvivoGen). Cells were incubated in the dark with shaking at 80 rpm, 37 °C, 5% CO₂ and 80% humidity using orbital Climo Shaker ISF1-X (Kuhner Shaker, Inc.). The starting medium volumes were 20 mL in the experiment shown in Fig. 1 and 30 mL in the experiments shown in Figs. 2, 3, 4, 5 and 6. Up to 1.5 mL of cell culture medium were taken for assessment every 24 h after seeding the cells.

Kinetic parameters

The specific growth, production and consumption rates were calculated as follows (Omasa et al. 1992). Recombinant protein concentrations of the cell supernatants were determined by Octet QKe (Pall ForteBio LLC., Menlo Park, CA, USA). Protein A biosensors (Pall ForteBio LLC.) and Protein A calibrator (Pall ForteBio LLC.) were used to measure the IgG1 concentration. Glucose, lactate, glutamine, glutamic acid and ammonium ion concentrations were determined by BioProfile 400 (Nova Biomedical Corp., Waltham, MA, USA).

Fig. 1 The effect of EGCG on antibody production quantity. EGCG was added to the medium on the first day of culture, and cultures were continued until cell viability fell to less than 60% after reaching the maximum cell density in batch cultures. The final IgG1 concentrations in the supernatants of 0, 5, 7 or 8 μ M EGCG-treated IgG1-producing cell cultures were determined by Octet QKe. Values are expressed as mean $\pm$ standard deviation ($n = 3$), *** $p < 0.001$ versus 0 μ M EGCG

N-glycan analysis of IgG1

IgG1 purification from the cell culture supernatant and labeling of released N-glycans with 2-aminobenzamide were performed by EZGlyco (Sumitomo Bakelite Co. Ltd., Tokyo, Japan). Prepared samples were analyzed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) at Sumitomo Bakelite Co. Ltd.

Statistical analysis

Data are presented as mean $\pm$ standard deviation. The unpaired Student's two-tailed t -test was used to compare the difference between two groups. Analysis of variance (ANOVA) was used to compare the difference among four groups. As a post hoc test, multiple comparisons with Scheffe's method were done.

Results

The effect of EGCG on antibody production level

EGCG was added to the medium at a final concentration of 0, 5, 7 or 8 μ M on the first day of culture for the productivity assessment. The antibody concentration in the supernatants of EGCG-treated cells was measured when cell viability fell to less than 60% after reaching the maximum cell density in batch cultures. The culturing period was extended in the cultures with 7 μ M EGCG or 8 μ M EGCG. Namely, cell culture lasted 7 days with 0 or 5 μ M EGCG, 9 days with 7 μ M EGCG, and 10 days with 8 μ M EGCG. There was no effect on the final IgG1 concentration in the culture medium containing 5 μ M EGCG (Fig. 1). Conversely, in the culture media containing 7 μ M EGCG or 8 μ M EGCG, the final IgG1 concentrations significantly increased compared with the vehicle control (Fig. 1).

The effects of EGCG on cell viability and cell growth

The effects of 8 μ M EGCG on cell viability and cell growth were determined. Cell viability and viable cell density were measured every 24 h after cell seeding. The lifetime of cells increased in the medium

Fig. 2 The effects of EGCG on cell viability and cell growth. Cell viability (**a**) and viable cell density (**b**) of cells treated with 0 μM EGCG (filled circles) or 8 μM EGCG (open circles) during batch cultures are shown. Total/viable cell concentrations were determined by the automated cell analyzer, Vi-cell XR. Values are expressed as mean ± standard deviation

($n = 3$), * $p < 0.05$ and *** $p < 0.001$ versus 8 μM EGCG. (**c**) The specific growth rates of 0 or 8 μM EGCG-treated cells were calculated based on the cell viability and viable cell density data. Values are expressed as mean ± standard deviation ($n = 3$), ** $p < 0.01$ versus 0 μM EGCG

Fig. 3 The effect of EGCG on specific antibody production rate. **a** IgG1 concentrations determined by Octet QKe in 0 μM EGCG (filled circles) or 8 μM EGCG (open circles)-treated cell culture supernatants during batch cultures are shown. Values are expressed as mean ± standard deviation ($n = 3$), * $p < 0.05$ and

*** $p < 0.001$ versus 8 μM EGCG. **b** The specific IgG1 production rates of 0 or 8 μM EGCG-treated cells were calculated based on the IgG1 concentrations and viable cell density data. Values are expressed as mean ± standard deviation ($n = 3$), * $p < 0.05$ versus 0 μM EGCG

containing 8 μM EGCG, even though the cell viability was slightly lower than for the control during the first few days (Fig. 2a). The specific cell growth rate in the medium containing 8 μM EGCG was lower than in the medium without EGCG (Fig. 2c). Furthermore, EGCG did not affect the maximum cell density (Fig. 2b).

The effect of EGCG on the specific antibody production rate

IgG1 concentrations in the cell culture supernatants were determined every 24 h after cell seeding during batch cultures. During the first 7 days, the amount of IgG1 in the supernatants with 8 μM EGCG tended to

Fig. 4 The effect of EGCG on cell metabolism. Glutamine (circles) and ammonium ions (triangles) (a), glucose (circles) and lactate (triangles) (c), and glutamic acid (e) concentrations determined by BioProfile 400 in 0 μM EGCG (filled symbols) or 8 μM EGCG (open symbols)-treated cell culture supernatants during batch cultures are shown. Values are expressed as

mean ± standard deviation (n = 3). The glutamine (b) and glucose consumption rates (d) of 0 or 8 μM EGCG-treated cells were calculated based on the glutamine or glucose concentrations and viable cell density data. Values are expressed as mean ± standard deviation (n = 3), **p* < 0.05 versus 0 μM EGCG

be lower than the amount in the control culture supernatants because of lower cell numbers (Fig. 3a). However, the amount of secreted IgG1 in the supernatants with 8 μM EGCG was higher than in the supernatants without EGCG because of the longer lifetime of the cells (Fig. 3a). The final yield of recombinant IgG1 in the supernatants of 8 μM EGCG-treated cells increased by almost 50% compared with untreated cell culture supernatants when cell viability decreased to less than 60% after reaching the maximum cell density in batch cultures (Fig. 3a). More

importantly, the production rate per cell was higher in 8 μM EGCG-treated cells than in the control (Fig. 3b).

The effect of EGCG on cell metabolism

The concentration of glucose, lactate, glutamine, glutamic acid and ammonium ions was also determined in cell culture supernatants every 24 h after cell seeding during batch cultures. A lower glutamine consumption rate per cell was observed in medium containing 8 μM EGCG (Fig. 4a, b). However, there

A 0 μ M EGCG

peak	obsd <i>m/z</i>	calcd <i>m/z</i>	ion species	Estimated glycan composition ('GlycoMod' database)
1	899.43	-	[M-H] ⁻	N/A
2	790.27	790.30	[M-2H] ²⁻	(HexNAc) ₂ (Deoxyhexose) ₁ + (Man) ₃ (GlcNAc) ₂ : G0F
3	1353.46	1353.49	[M-H] ⁻	(Hex) ₂ + (Man) ₃ (GlcNAc) ₂ : MAN5
4	798.78	-	[M-H] ⁻	N/A
5	871.30	871.33	[M-2H] ²⁻	(Hex) ₁ (HexNAc) ₂ (Deoxyhexose) ₁ + (Man) ₃ (GlcNAc) ₂ : G1F
6	871.30	871.33	[M-2H] ²⁻	(Hex) ₁ (HexNAc) ₂ (Deoxyhexose) ₁ + (Man) ₃ (GlcNAc) ₂ : G1F
7	855.45	-	[M-H] ⁻	N/A
8	952.32	952.35	[M-2H] ²⁻	(Hex) ₂ (HexNAc) ₂ (Deoxyhexose) ₁ + (Man) ₃ (GlcNAc) ₂ : G2F
9	1097.86	1097.90	[M-2H] ²⁻	(Hex) ₂ (HexNAc) ₂ (Deoxyhexose) ₁ (NeuAc) ₁ + (Man) ₃ (GlcNAc) ₂ : G2FS1
10	899.48	-	[M-H] ⁻	N/A

B 8 μ M EGCG

peak	obsd <i>m/z</i>	calcd <i>m/z</i>	ion species	Estimated glycan composition ('GlycoMod' database)
1	717.24	717.27	[M-2H] ²⁻	(HexNAc) ₂ + (Man) ₃ (GlcNAc) ₂ : G0
2	790.27	790.30	[M-2H] ²⁻	(HexNAc) ₂ (Deoxyhexose) ₁ + (Man) ₃ (GlcNAc) ₂ : G0F
3	901.48	-	[M-H] ⁻	N/A
4	900.48	-	[M-H] ⁻	N/A
5	871.30	871.33	[M-2H] ²⁻	(Hex) ₁ (HexNAc) ₂ (Deoxyhexose) ₁ + (Man) ₃ (GlcNAc) ₂ : G1F
6	871.30	871.33	[M-2H] ²⁻	(Hex) ₁ (HexNAc) ₂ (Deoxyhexose) ₁ + (Man) ₃ (GlcNAc) ₂ : G1F
7	899.48	-	[M-H] ⁻	N/A
8	952.32	952.35	[M-2H] ²⁻	(Hex) ₂ (HexNAc) ₂ (Deoxyhexose) ₁ + (Man) ₃ (GlcNAc) ₂ : G2F
9	1097.86	1097.90	[M-2H] ²⁻	(Hex) ₂ (HexNAc) ₂ (Deoxyhexose) ₁ (NeuAc) ₁ + (Man) ₃ (GlcNAc) ₂ : G2FS1
10	943.51	-	[M-H] ⁻	N/A

Fig. 5 The effect of EGCG on N-linked glycosylation. **a, b** The glycan profiles of IgG1 purified from cell culture supernatants on day 6 of batch culture with 0 μ M EGCG (**a**) and on day 8 of batch culture with 8 μ M EGCG (**b**) were analyzed by HPLC–MS, to determine the glycan structures. The estimated glycan compositions of the top ten peaks are shown. Glycan compositions were estimated by the GlycoMod tool, and those recorded in the UniCarbKB database were extracted

was no significant change in the glucose consumption rate between the two conditions (Fig. 4c, d). The glutamic acid concentration remained approximately constant (Fig. 4e).

The effect of EGCG on N-linked glycosylation

We then assessed whether EGCG affects the N-linked glycosylation of IgG1. The glycan profile and the structures of the N-linked glycan detected on IgG1 were analyzed by HPLC–MS. The IgG1 glycan structures of G0F, G1F, G2F, G2FS1 and Man5 were detected among the top ten peaks in the control treatment (Fig. 5a). In the 8 μ M EGCG treatment, the IgG1 glycan structures of G0F, G1F, G2F, G2FS1 and G0 were detected among the top ten peaks (Fig. 5b). The Man5 glycoform of IgG1 was only detected in the control, whereas the G0 glycoform of IgG1 was only detected in the 8 μ M EGCG treatment (Fig. 5a, b). There were no significant differences between the two conditions, indicating that the N-linked glycosylation pattern of IgG1 was not greatly affected by 8 μ M EGCG.

The effect of the timing of EGCG addition on antibody production quantity

To evaluate the effect of the timing of EGCG addition on cell growth and antibody productivity, three conditions were compared (Fig. 6a). In the first one EGCG was added at the beginning of culture, in the second it was added on day 4 of culture, and in the third it was added on days 0 and day 4 (Fig. 6a). The antibody concentration in the supernatant of EGCG-treated cells was measured when cell viability fell to less than 60% after reaching the maximum cell density in batch cultures. The culture period was extended only when 8 μ M EGCG was added from the beginning compared with the control. Namely, the cell culture lasted 10 days when EGCG was added at the beginning, whereas it lasted 7 days when EGCG was added

Fig. 6 The effect of the timing of EGCG addition on antibody production level. **a** Schematic diagram of the experiment. EGCG was added at the beginning or after 4 days of culture, once or twice. **b** The final IgG1 concentrations in cell culture supernatants of EGCG-treated IgG1-producing cells when cell viability fell to less than 60% after reaching the maximum cell density in batch cultures were determined by Octet QKe. Values are expressed as mean $\pm$ standard deviation ($n = 3$), *** $p < 0.001$ versus the other three groups

on day 4 of culture, on days 0 and 4, or when it was not added. There was no effect on the final IgG1 concentration in the culture medium when EGCG was added on day 4 of culture or when it was added on days 0 and 4 (Fig. 6b). The final IgG1 concentration in the culture medium was significantly higher than that in the control only when 8 μ M EGCG was added at the beginning (Fig. 6b).

Discussion

In this study, we showed a new approach for controlling the cell cycle phase using EGCG, a natural compound used during therapeutic antibody production by CHO cells. We think that 8 μ M EGCG did not cause a dramatic reduction in cell metabolism because it did not induce a significant change in the glucose consumption rate. Though the reason why the glucose consumption rate did not change under different proliferation rate remains unclear, the surplus energy generated due to reduced proliferation may have been used for antibody production. However, the glutamine

consumption rate was lower in the 8 μM EGCG-treated cells. EGCG has antioxidant and scavenger functions. Decreased glutamine concentrations are found during catabolic stress (Matés et al. 2002), hence it may be suggested that there is less stress in the culture environment with the antioxidant, EGCG, because glucose consumption rates are constant. Longer life time was probably caused by delayed nutrient depletion in batch cultures.

Proliferation control strategies to increase productivity are typically performed in the mid- or late-phases of exponential growth during culturing (Kumar et al. 2007). However, under our conditions, EGCG addition was not effective when it was added at the intermediate point of culture, but it was effective when it was added on the first day of culture. One benefit of this is that it is simpler and more uniform to add a chemical just at the beginning of culturing rather than in the middle of the culture period. Secreted metabolites of cells may affect the growth inhibitory effect of EGCG, when it was added at the intermediate point. In addition, it was not effective to add EGCG in portions at two time points. It has been reported that in cultured normal keratinocytes, EGCG treatment for 5 days significantly increased their proliferation in a dose-dependent manner up to 1 μM , but not with 10 μM EGCG (Chung et al. 2003). This report also stated that the phosphorylation of Erk and PKB/Akt increased by EGCG at 0.5 μM , but not at 50 μM in normal keratinocytes (Chung et al. 2003). In our experiments, 4 μM EGCG and 5 μM EGCG treatments tended to enhance cell growth, in contrast to 7 and 8 μM EGCG treatments, which slowed cell growth (data not shown). Furthermore, treatment with more than 10 μM EGCG resulted in lower cell growth and a higher percentage of cell apoptosis (data not shown), which is similar to previously published studies (Ahn et al. 2003; Chung et al. 2003). These results suggest that the effect of EGCG on cell proliferation is concentration-dependent.

EGCG also inhibits DNA methyltransferases (DNMTs) activity (Berletch et al. 2008; Brueckner and Lyko 2004; Rajavelu et al. 2011). DNA methylation of transgene promoters leads to loss in productivity of monoclonal antibody-producing CHO cell lines (Osterlehner et al. 2011; Yang et al. 2010). It has been reported that 5-Aza-2'-deoxycytidine, a DNA methylase inhibitor, partially restored transgene activation (Choi et al. 2005; Yang et al. 2010). Protein

production stability during long-term culture is a major issue in the biopharmaceutical industry, and one of the main concerns in this area is CpG methylation in the promoter region of the transgene (Barnes et al. 2003; Wurm 2004). Thus, EGCG effects on DNMTs inhibition may improve production stability. However, such activity against DNMTs may modulate the transcription of other genes through an epigenetic pathway, which will also affect protein glycosylation. In addition, different culture conditions lead to different glycosylation patterns of the IgG monoclonal antibody (Patel et al. 1992). The N-linked glycosylation of the biopharmaceutical human IgG, which affects antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities, is an important determinant of the quality of recombinant IgG1 as an antibody preparation (Arnold et al. 2007; Eon-Duval et al. 2012; van Berkel et al. 2009). Therefore, the EGCG effect on IgG1 N-glycosylation was investigated. The Man5 glycoform of IgG1 was not detected in the culture with 8 μM EGCG, while it was detected in the control. High-mannose glycans such as Man5 on the Fc region of the therapeutic IgG antibodies increase the serum clearance in humans and are considered as undesirable (Goetze et al. 2011). There were no other glycoform changes by 8 μM EGCG addition, hence we considered that 8 μM EGCG has no influence on the antibody quality. Thus, cell cycle control by 8 μM EGCG is safe and worth considering to enhance productivity and expand the total amount of IgG1 production in CHO-K1 cell lines.

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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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