Enhancement of antibody production by the addition of Coenzyme-Q₁₀

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Abstract Recently, there has been a growing demand for therapeutic monoclonal antibodies (MAbs) on the global market. Because therapeutic MAbs are more expensive than low-molecular-weight drugs, there have been strong demands to lower their production costs. Therefore, efficient methods to minimize the cost of goods are currently active areas of research. We have screened several enhancers of

specific MAb production rate (SPR) using a YB2/0 cell line and found that coenzyme-Q₁₀ (CoQ₁₀) is a promising enhancer candidate. CoQ₁₀ is well known as a strong antioxidant in the respiratory chain and is used for healthcare and other applications. Because CoQ₁₀ is negligibly water soluble, most studies are limited by low concentrations. We added CoQ₁₀ to a culture medium as dispersed nanoparticles at several concentrations (Q-Media) and conducted a fed-batch culture. Although the Q-Media had no effect on cumulative viable cell density, it enhanced SPR by 29%. In addition, the Q-Media had no effect on the binding or cytotoxic activity of MAbs. Q-Media also enhanced SPR with CHO and NS0 cell lines by 30%. These observations suggest that CoQ₁₀ serves as a powerful aid in the production of MAbs by enhancing SPR without changing the characteristics of cell growth, or adversely affecting the quality or biological activity of MAbs.

Keywords Antioxidant · Coenzyme-Q₁₀ · Chinese hamster ovary (CHO) · 8-hydroxy-2'-deoxyguanosine · Monoclonal antibodies · NS0 · Specific production rate · YB2/0 · Enhancer · Productivity

Abbreviations

MAbs	Monoclonal antibodies
CoQ ₁₀	Coenzyme-Q ₁₀
Q-Media	Culture media supplemented with dispersed nanoparticles of Q ₁₀

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8OHdG	8-hydroxy-2'-deoxyguanosine
SPR	Specific MAb production rate (pg cell ⁻¹ d ⁻¹)

Introduction

Recently, more than twenty therapeutic monoclonal antibodies (MAbs) and related proteins have been launched in the market (Shukla and Thömmes 2010; Walsh 2006). This success is a double-edged sword, however, because it leads to pressure on the pharmaceutical economy. Minimizing the cost of goods and maximizing antibody activity are therefore active areas of research in the development of MAbs for therapeutic use (Shields et al. 2002; Shinkawa et al. 2003; Wurm 2004). There is also great interest in establishing a stable global supply of MAbs.

Several approaches have been explored to improve MAb production in cell cultures. Over the past decade, various approaches such as genetic engineering, clone selection, medium optimization, and reactor engineering have dramatically improved production (Takenouchi and Sugahara 2003). The addition of an enhancer has also proven effective. In the development of biotherapeutics, when an appropriate cell bank is established, we want to maintain use of the same cell bank to avoid risks derived from shifting characterization. The advantage of adding enhancers is the flexibility that can be applied at any development phase. Such enhancers should meet the following requirements: (a) be safe for operators and patients, (b) maintain the quality of the final product, (c) be cost-effective, and (d) be easy to handle. With these requirements in mind, the following enhancers have been suggested: butyrate (De Leon Gatti et al. 2007; Jiang and Sharfstein 2008; Mimura et al. 2001; Oh et al. 1993, 2005), caspase inhibitor (Arden et al. 2007), carnosine (Yegorov et al. 2007), glycerol (Liu and Chen 2007), nucleosides (Carvalho et al. 2003), linoleic acid (Butler et al. 1999), lipopolysaccharide (Martin-Lopez et al. 2007), dimethyl sulfoxide (Ling et al. 2003; Tsao et al. 2001), rapamycin (Balcarcel and Stephanopoulos 2001), retinoic acid (Inoue et al. 2000), sericin (Terada et al. 2002), spermine (Miyazaki et al. 1998), various sugars (Coroadinha et al. 2006), valproic acid (Backliwal et al. 2008; Konno et al. 2006), and vitamin A (Inoue et al. 1999) (summarized in Table 1).

Coenzyme-Q is a biosynthesized quinone that occurs widely in living organisms such as yeasts, plants, and animals and as such is also known as ubiquinone (ubiquitously occurring quinone). In higher organisms, including humans, this compound has 10 isoprenoid units in its side chain and is therefore named Coenzyme-Q₁₀ (CoQ₁₀; Hathcock and Shao 2006). CoQ₁₀, also known as Ubiquinone-Q₁₀, is widely used as a therapeutic agent, a dietary supplement, an ingredient in cosmetic products, and various other products (Kitano et al. 2004; Müller et al. 2007; Ochiai et al. 2007). CoQ₁₀ is a lipid-soluble compound located in the inner mitochondrial membrane where it functions as a part of the electron transport chain as well as a strong endogenous lipophilic antioxidant (Turunen et al. 2004). CoQ₁₀ is commonly used as a dietary supplement, with the rationale that increasing intake of the nutrient boosts cellular metabolism, particularly in cells with energy deficiencies (Mancuso et al. 2010). The effect of CoQ₁₀ has been tested on various diseases such as diabetes (Hodgson et al. 2002), myocardial infarction (Singh et al. 1998; Soja and Mortensen 1997), angina (Kogan et al. 1999), and Parkinson's disease (Shults et al. 2004), as well as in anti-breast cancer clinical trials (Portakal et al. 2000). It is also used as adjunct therapy in patients following cardiac surgery (Chello et al. 1996; Hathcock and Shao 2006).

CoQ₁₀ was found to inhibit cell growth and induce apoptosis in HeLa cells (Gorelick et al. 2004), but to decrease apoptosis in human T-acute lymphatic leukemia cells by inhibiting the activity of caspase-3 (Navas et al. 2002). Therefore, the effects of CoQ₁₀ on apoptosis remain controversial. CoQ₁₀ was also shown to promote the growth of many cell lines, including HeLa, HL-60, mouse fibroblast, and bovine embryo cells (Stojkovic et al. 1999; Sun et al. 1992a, 1995). Although CoQ₁₀ as an ingredient in various media appears to stimulate growth, there have been no reports that have directly analyzed this effect on SPR. In this paper, we report the effects of CoQ₁₀ on YB2/0, CHO, and NS0 cell lines.

Materials and methods

Materials

CoQ₁₀ (Kyowa Hakko Bio Co., Ltd., Tokyo, Japan) and Tween-80 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) at a 1:2 weight ratio were dissolved

Table 1 Reported enhancers of protein production in animal cell lines

Enhancer	Range/optimal	Product	Effect ^a	Cell line	Reference
AMP/nucleotides, nucleosides, and bases	1 mM	Human placental alkaline phosphatase enzyme (SEAP)	3-fold in SPR	CHO-K1	Carvalho et al. (2003)
Carnosine	1 mM	Factor VII	2-fold in SPR	CHO-K1	Yegorov et al. (2007)
	0.3 mM	Factor VII	2.5-fold in SPR	BHK	
	20 mM	Telomerized cells	2.5-fold in total number cells	Telomerized cells	
Chemical caspase inhibitors	5–50 μ M/5 μ M	MAB IgG	1.2-fold in MAb titer	CHO-K1, HEK-293	Arden et al. (2007)
Dimethyl sulfoxide (DMSO)	0–1.5%/0.2% (v/v)	MAB	2-fold in SPR	Hybridoma clone 19	Ling et al. (2003)
Dimethyl sulfoxide (DMSO)	0–5 mM/1 mM	Adenoviral vector	3-fold in Virus production	HEK293	Tsao et al. (2001)
Sodium butyrate,	0–5 mM/0.5 mM	Macrophage-colony stimulating factor (M-CSF)	1.5-fold	CHO	Liu and Chen (2007)
Ethyl alcohol,	0–200 mM/100 mM		1.8-fold		
N-acetyl-l-cysteine	0–5 mM/2.5 mM		1.8-fold		
Glycerol	0–2.0%/1%	IgM	1.4-fold in titer	Human–human hybridoma HB4C5	Takenouchi and Sugahara (2003)
Lactate dehydrogenase (LDH)	320 μ g/mL		12.4-fold		
Linoleic acid	25–50 μ M/25 μ M	MAB (IgG1)	–	Hybridoma (CC9C10)	Butler et al. (1999)
Long TM R3 IGF-I	–	MAB	1.2-fold in titer	CHO	Kim et al. (2005)
Triiodothyronine (T ₃)	–	IgG2a to human immunodeficiency virus (HIV) glycoprotein 120 (gp120).	1.2-fold in titer	Mouse-mouse B cell hybridoma line (CD40 and CD19-deficient expression)	Martin-Lopez et al. (2007)
Lipopolysaccharide (LPS)	60 pg cell ⁻¹		3-fold in SPR		
Pyruvate	0.02 M	Anti-ribonuclease A antibody	1.4-fold in SPR	Hybridoma 3A21	Omata et al. (2010)
Malate	0.005 M	MAB	1.1-fold in SPR	Hybridomas CRL 1606	Balcarcel and Stephanopoulos (2001)
Citrate	0.05 M		1.1-fold in SPR		
Rapamycin	100 nM		0.25 to 0.56 g/L, 1.24-fold		
Retinoic acid	10 ⁻⁷ M	IgG	8-fold	Human–human hybridoma BD9	Inoue et al. (2000)

Table 1 continued

Enhancer	Range/optimal	Product	Effect ^a	Cell line	Reference
Small molecule enhancers	0.5 mM	MAb	1.6-fold	CHO	Allen et al. (2008)
Sodium butyrate (NaBu) + N-acetylcysteine (NAC)	1 mM NaBu and 8 mM NAC	IFN-beta	2-fold	CHO	Oh et al. (2005)
Sodium butyrate	0–5 mM/2 mM	Mouse/human chimeric IgG3	2- to 4-fold	CHO-K1	Mimura et al. (2001)
Sodium butyrate	5 mM	Humanized IgG	1- to 4-fold	CHO 9 clones	Jiang and Sharfstein (2008)
Spermine	7.3 mM	IgM, IgG	6-fold	Human-human hybridoma, HB4C5	Miyazaki et al. (1998)
Sugar sources: glucose, galactose, sorbitol, and fructose	Glc 25–140 mM Frc 83–140 mM	MoMLV-derived recombinant retroviral vectors	14-fold	Retroviral producer cell line	Coradinha et al. (2006)
Valproic Acid	100 µM–20 mM/500 µM	MAb	4-fold, 1.5-fold	CHO-DG44	Backliwal et al. (2008)
Valproic Acid	100 µM	MAb	3-fold	CHO	Konno et al. (2006)
Vitamin A acetate	1 µg/L	Human MAb AE6F4	0.9- to 2.9-fold	Hybridoma	Inoue et al. (1999)

^a Recalculated % to fold: 20% to 1.2-fold

completely in ethanol. The solution was heated to remove ethanol, diluted with water, and filtered with a 0.2-µm filter to produce a CoQ₁₀/Tween-80 stock solution. SANOMITTM Q₁₀ (MSE, Hamburg, Germany) was also used as a possible enhancer of SPR because of its easy handling compared with various other commercial CoQ₁₀ preparations. SANOMITTM Q₁₀ is a dispersion of nanoparticles (diameter <50 nm) of CoQ₁₀ in water and is useful for preparing stable liquid–liquid dispersions of various CoQ₁₀ concentrations. Hereafter, we refer to the media supplemented with SANOMITTM Q₁₀ as Q-Media, irrespective of the original composition of the media (Konno et al. 2001). We also tested other enhancer candidates including l-glutathione, alpha-tocopherol, l-ascorbic acid, 2-mercaptoethanol, and 2,3-dimethoxy-5-methyl-p-benzoquinone (Sigma Chemical. Co., Ltd., MO, USA).

Cell culture

In this study, the anchorage-dependent NS0 cell lines (RCB0213; Riken, Ibaraki, Japan) were cultivated in 225 cm² T-flasks (Asahi Glass, Tokyo, Japan). CHO (Urlaub and Chasin 1985) and YB2/0 cell lines (ATCC 1662) were cultivated in 250 mL shaker flasks (431406; Corning, NY, USA), or in 1 L bioreactors (ABLE, Tokyo, Japan; Ogawa et al. 1999). CHO, YB2/0, and NS0 cell lines expressing proprietary recombinant mouse/human chimeric IgG1 antibody were cultured in Q-Media. Different Q-Media were prepared from ExCellTM 302 (SAFC-Bioscience, St. Louis, MO, USA), RPMI-1640 Medium, Hybridoma-SFM (serum-free medium, with added BSA and transferrin), or CD-Hybridoma (animal-derived, protein-free; Life Technologies, Carlsbad, CA, USA) depending on the cell line used. The cultures were inoculated at a density of 2×10^5 cells mL⁻¹ and cultivated at 37 °C with occasional feeding with serum-free Iscove's Modified Dulbecco's Medium until the decline phase. In the culture of 1 L bioreactors, the culture was maintained at pH 7.1 by CO₂ gas overlay and 1 mM Na₂CO₃ alkaline solution at 50% DO by sparger O₂ gas.

Cell culture monitoring

The number of viable and dead cells was determined using a CEDEXTM counter (Innovatis AG, Bielefeld, Germany) by the trypan blue dye exclusion method.

Off-line measurements of culture glucose and lactate were carried out using an YSI 2700 bioanalyzer (Yellow Springs Inc., OH, USA). The concentration of MABs in the culture supernatant was determined by HPLC using a Protein A column. The 8-OHdG concentration was quantified using an ELISA kit (8-OHdG check; Japan Institute for the Control of Aging, Shizuoka, Japan).

ADCC activity

Antibody-dependent cell-mediated cytotoxicity (ADCC) was determined by ^{51}Cr release assay, as previously reported (Nakamura et al. 1999).

Equations

$$\text{Viability (\%)} = \frac{\text{viable cells (cells/mL)}}{\text{total cells (cells/mL)}} \times 100$$

$$\text{Cumulative cell density (cells/mL} \times \text{d)} = \sum [\text{viable cells}_2 + \text{viable cells}_1 (\text{cells/mL})] \times [\text{time}_2 - \text{time}_1 (\text{d})] / 2$$

$$\text{Specific monoclonal antibody production rate} \\ (\text{MAB} - \text{pg/cell/d}) = \text{MAB}(\text{mg/L}) / \text{CCD} \\ (\text{cells/mL} \times \text{d})$$

Results and discussion

CoQ₁₀ enhanced specific MAB production rate

When an enhancer is used as an additive to culture media for the production of commercial MABs,

consideration must be given to various factors including safety, cost, stability, stable supply, simple usage, patents, and regulations. With these restrictions in mind, we screened various compounds, which can be classified into groups related to the following: (1) nutrition, (2) anti-oxidization, (3) electron transport, (4) growth inhibition or cell cycle, and (5) stress response. In preliminary experiments using YB2/0 cells in 250-mL flasks, media supplemented with 50 μM CoQ₁₀ (dissolved with the aid of Tween-80) exhibited marked enhancement of SPR compared with media supplemented with only Tween-80. Such enhancement (1.26-fold) was also observed for the YB2/0 culture in a 1 L bioreactor (Fig. 1B). Although CoQ₁₀ dissolved with the aid of Tween-80 exhibited enhanced SPR, CoQ₁₀ concentration was limited (<100 μM), and dissolution of CoQ₁₀ required tedious procedures. These problems were circumvented by the use of a stable dispersion of CoQ₁₀ nanoparticles (SANOMITTM Q₁₀); CoQ₁₀ concentration was easily elevated to as high as 500 μM , and a 1.28-fold increase in SPR could be obtained (Fig. 2). Although the promotion of cell growth by CoQ₁₀ (typically at 30 μM) has been reported in several cell lines (Stojkovic et al. 1999; Sun et al. 1992b, 1995), our results are the first to show the enhancement of SPR by CoQ₁₀.

CoQ₁₀ did not affect oxidative response in fed-batch cultures

CoQ₁₀ is known as a strong antioxidant, and 8-OHdG is known as a marker for oxidative DNA damage (Takane

Fig. 1 Effects of CoQ₁₀ on SPR. The YB2/0 cell line was cultured in medium supplemented with CoQ₁₀ (dissolved with Tween-80; closed circle) and with Tween-80 alone (control; open circle) in a 1 L reactor (ABLE, Tokyo, Japan).

A Comparison of cell growth, **B** comparison of SPR. Two other independent experiments with similar conditions exhibited similar results

Fig. 2 Effects of Q-Media (containing 500 μM CoQ₁₀ in Hybridoma-SFM) on SPR. The YB2/0 cell line (expressing recombinant mouse/human chimeric IgG1 antibody) was cultured in Q-Media (closed circle) or in regular medium (open circle) in a 1 L bioreactor (ABLE, Tokyo, Japan). **A** Time course of 8-OHdG concentration, **B** comparison of cell growth, and **C** amount of MAb vs. cumulative cell density (its slope corresponds to SPR). Six other independent experiments with similar conditions exhibited similar results

et al. 2002). We evaluated the change in 8-OHdG concentrations in fed-batch cultures to examine the antioxidant effects of Q-Media (containing 500 μM CoQ₁₀). Although SPR was enhanced by the Q-Media, the 8-OHdG concentrations (and cumulative cell density) did not change (Fig. 2). To confirm that the enhancement of SPR by CoQ₁₀ was not ascribed to the antioxidant effect of CoQ₁₀, we analyzed the effects of other antioxidants. None of the following compounds, namely, glutathione ($\leq 1,000$ μM), alpha-tocopherol ($\leq 5,000$ μM), proanthocyanidin B2 ($\leq 1,000$ μM), uric acid ($\leq 1,000$ μM), ascorbic acid ($\leq 1,000$ μM), or 2-mercaptoethanol ($\leq 5,000$ μM), enhanced SPR as expected (data not shown).

Q-Media enhanced SPR for CHO and NS0 cell lines

To examine whether the enhancement of SPR by Q-Media is a general phenomenon, we analyzed the effects of Q-Media on two additional host cell lines commonly used in MAb production—CHO and NS0. SPR was also enhanced in CHO and NS0 cell lines by 28.8 and 31.9%, respectively, and in YB2/0 by 29.0%, which suggests that Q-Media is a versatile agent for enhancing SPR. Although a 20% increase in SPR may not seem notable, this degree of enhancement is most welcome. To illustrate the potential

effectiveness of such enhancement, a 20% increase in SPR could reduce the annual cost (\$65 million) of obtaining 250 kg of MAbs with a yield of 1,000 mg/L in a 10,000 L plant (Werner 2004) by \$6 million. In fact, increases of 10%–20% in SPR of various MAbs have already been reported (Arden et al. 2007; Kim et al. 2005; Omasa et al. 2010).

Activities of MAbs were not changed by culturing in Q-Media

Because strict comparability to original MAbs is required when production conditions are changed, and because MAbs produced in YB2/0 cells have been known to exhibit high ADCC activities, we analyzed antigen binding and ADCC activities of MAbs expressed in YB2/0 cells that were cultured in Q-Media and regular media. MAbs produced in both media exhibited indistinguishable results for both activities (Fig. 3), which warrants the safe use of Q-Media for enhancing SPR.

Conclusion

In this study, we found that supplementing culture media with CoQ₁₀ widely enhances the production rate of MAbs in YB2/0, CHO, and NS0 cells without

Fig. 3 Comparison of MABs produced in Q-Media and regular medium. MABs expressed by YB2/0 cells cultured in Q-Media and regular medium were separately purified and their activities to bind to their antigen (panel **A**) and kill their target cells (ADCC, panel **B**) were analyzed by ELISA and ^{51}Cr -release assays, respectively

affecting cell growth or MAb activities such as antigen binding and ADCC. Although the mechanisms of enhancement are unclear, CoQ₁₀ will be useful for the efficient production of pharmaceutically useful MABs.

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