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REVIEW

Proliferation control strategies to improve productivity and survival during CHO based production culture

A summary of recent methods employed and the effects of proliferation control in product secreting CHO cell lines

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Abstract Chinese Hamster Ovary cells are the primary system for the production of recombinant proteins for therapeutic use. Protein productivity is directly proportional to viable biomass, viability and culture longevity of the producer cells and a number of approaches have been taken to optimise these parameters. Cell cycle arrest, particularly in G1 phase, typically using reduced temperature cultivation and nutritional control have been used to enhance productivity in production cultures by prolonging the production phase, but the mechanism by which these approaches work is still not fully understood. In this article, we analyse the public literature on proliferation control approaches as they apply to production cell lines with particular reference to what is known about the mechanisms behind each approach.

Keywords CHO · Proliferation control · Productivity · Temperature · Cell cycle · Nutrients

Introduction

Chinese Hamster Ovary (CHO) cell lines were established in 1957 by Puck and colleagues at the Department of Biophysics, University of Colorado Medical School in Denver, USA from ovarian biopsy of an adult Chinese hamster (Puck et al. 1958). CHO are the preferred system for large scale production of recombinant proteins for therapeutic use as they are fast growing; can be readily transfected and can perform complex post-translational modifications required for biological activity. They have also been used safely for the production of recombinant therapeutics for many years and as such are a trustworthy system.

Over the last two decades, significant improvements have been made in recombinant protein productivity in CHO. In 1986, a typical 7 day CHO batch process achieved a maximal density of ~2×10^6 cells/ml with a specific productivity of ~10 pg/cell/day facilitating a final product titre of ~50 mg/l. Today however, cells can achieve >10×10^6 cells/ml with specific productivities of ~90 pg/cells/day and product titres in excess of 5 g/l (Wurm 2004). These improvements also allow maintenance of high viability for up to 3 weeks.

Recombinant protein yields from CHO fermentations are directly correlated with cell number and culture longevity. Cell growth in standard production culture systems can be divided into four phases i.e. lag, log, stationary and decline phase. Long lag phases are undesirable, therefore high growth rates are essential at the beginning of

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a process to achieve sufficient cells for maximal production. Uncontrolled proliferation beyond a certain desired cell density is also undesirable due to nutrient and oxygen depletion, toxic metabolite accumulation, cell death, and degradation of the products (Al-Rubeai and Singh 1998; Zeng et al. 1998, Zeng and Deckwer 1999). Proliferation control strategies are typically implemented in the mid- or late-phases of exponential growth and have been shown to improve both the productivity and duration of production processes. Regulation of cell proliferation can generally be achieved through cell cycle arrest or through regulation of nutrients available in the culture media. A number of these strategies are discussed here.

The use of cell cycle arrest to increase recombinant protein productivity

Protein production is dependent on the phase of the cell-cycle and several genes such as those involved in ribosome biogenesis and protein translation are expressed highly in the G1 phase (Al-Rubeai and Emery 1990; Al-Rubeai et al. 1992; Moore et al. 1997; Fussenegger et al. 1998, 2000; Kaufman et al. 1999, 2001; Carvalhal et al. 2003; Ibarra et al. 2003; Yoon et al. 2003a, b; Fogolin et al. 2004; Bi et al. 2004; Trummer et al. 2006). Cells arrested at the end of G1-phase of cell cycle are metabolically more active and bigger in size than non-arrested cells (Carvalhal et al. 2003; Bi et al. 2004). For these reasons, the G1-phase of the cell cycle is considered the ideal time for increased production of recombinant proteins and G1 arrest has been used to increase the productivity in a number of commercially relevant cell lines such as hybridomas and CHO (Al-Rubeai and Emery 1990; Al-Rubeai et al. 1992; Moore et al. 1997; Fussenegger et al. 1998, 2000; Kaufman et al. 1999, 2001; Ibarra et al. 2003; Yoon et al. 2003a, b; Fogolin et al. 2004; Trummer et al. 2006). Some studies have reported the S phase as the optimal production phase (Lloyd et al. 2000; Fox et al. 2005), an example being the increased production of human interferon-γ (IFN-γ) upon increasing the percentage of CHO in S phase (Fox et al. 2005). This review will focus on G1 phase arrest approaches including (I) temperature shift to conditions of mild hypothermia, (II) cell engineering based approaches and (III) chemical approaches for cell cycle arrest.

(I) Temperature shift to conditions of mild hypothermia

Low culture temperature has been shown to reduce the growth rate, increase culture longevity and specific productivity in CHO cells expressing wide range of recombinant proteins (Furukawa and Ohsuye 1998, 1999; Kaufmann et al. 1999; Schatz et al. 2003; Yoon et al. 2003a, b, 2006; Fox et al. 2004; Fogolin et al. 2004, Bollati-Fogolin et al. 2005; Trummer et al. 2006). In this laboratory reducing the temperature of suspension CHO-K1 cells from 37 to 31°C during the latter stages of exponential growth resulted in an immediate cessation of proliferation. Cultures grown at 31°C achieved peak viable cell numbers that were almost 20% lower than in cells cultured for another 24 h at 37°C (Kumar et al. in preparation). A shift in the proportion of cells from the S to the G1 phase of the cell cycle has been observed at reduced temperatures which results in a state close to growth arrest and has been shown to improve productivity (Moore et al. 1997; Kaufamann et al. 1999; Hendrick et al. 2001; Yoon et al. 2003a, b; Fogolin et al. 2004; Trummer et al. 2006). Specific examples include the finding that 80% of secreted alkaline phosphatase (SEAP) producing and 76% of erythropoietin (EPO) producing CHO line accumulate in G0/G1 phase of cell cycle following reduction of the culture temperature to 30°C with associated increases in productivity (Kaufmann et al. 1999; Yoon et al. 2003b). More examples are given in Table 1. The quality of recombinant products, with regard to isoform pattern, sialic acid content, and in vivo biological activity, is maintained at reduced temperatures (Yoon et al. 2003b; Clark et al. 2004; Bollati-Fogolin et al. 2005). The mechanism whereby cells at lower temperatures improve productivity is still poorly understood although recent studies have demonstrated that reduced culture temperature invokes a coordi-

Table 1 Effect of reduced temperature on cell cycle and productivity in CHO

Contributor	Product expressed in CHO	Media type	Culture vessel	Reduced temperature (°C)	Cell cycle distribution in G0/G1 (%)	Productivity (fold change)	
						Qp	Y
Moore et al. (1997)	Chimeric Fab	DMEM/HAM's F-12 (1:1 mixture) + 1 g/l Pluronic F68, 0.2 ml/l antifoam emulsion	2L Bioreactor	30[a]	87	ND	ND
Kaufmann et al. (1999)	SEAP	FMX-8 medium + 10% FCS	T-25 flask	30[a]	80	+1.7	+3.4
Yoon et al. (2003a)	Anti-4-1BB antibody	IMDM + 10%FBS replaced with SF2 SFM in exponential phase and flask transferred to different temperatures	T-25 flask T-25 flask	30[b] 33[b]	77 67	NC NC	NC +3.9
Yoon et al. (2003b)	EPO	IMDM + 10%FBS replaced with SF2 SFM in exponential phase and flask transferred to different temperatures	T-75 flask T-75 flask	30[a] 33[a]	76 67	+5.6 +4	+2.5 –0.86
Fogolin et al. (2004)	hGM-CSF	ZKT-1 medium + 5% FCS	Spinner flasks	33[a]	65	+2.1	+2.3
Trummer et al. (2006)	Epo-Fc	DMEM/HAM's F-12 (1:1 mixture) + 0.58 g/l L-glutamine, an in-house developed protein-free supplement (proprietary formulation), 0.25% soy peptone, 0.1% Pluronic F68, and 0.096 mM MTX	0.5L Bioreactor	30[a]	65	+1.85	+1.3

Qp, cell specific productivity; Y, overall volumetric yield; ND, not determined; NC, no change

[a] Temperature shift from 37°C

[b] Continuous low temperature culture

nated response involving the cell cycle, transcription and translational machinery, and the arrangement of the cytoskeleton (Chuppa et al. 1997; Moore et al. 1997; Furukawa and Ohsuye 1998, 1999; Jorjani and Ozturk 1999; Hendrick et al. 2001; Yoon et al. 2003b; Fox et al. 2004, 2005; Baik et al. 2006; Al-Fageeh et al. 2006). Low culture temperature results in reduced metabolism (glucose/medium consumption, oxygen uptake, lactate production and ammonia production) and shear sensitivity which leads to delayed initiation of apoptosis and helps in extending the duration of the stationary/production phase (Moore et al. 1997; Chuppa et al. 1997; Furukawa and Ohsuye 1998, 1999; Jorjani and Ozturk 1999; Hendrick et al. 2001; Yoon et al. 2003a, b; Trummer et al. 2006; Wong et al. 2006). Reduced culture temperature has also been shown to increase levels of recombinant mRNA, either due to enhanced transcription of the recombinant gene of interest or increased mRNA stability (Hendrick et al. 2001; Yoon et al. 2003b; Fox et al. 2004, 2005).

For the above reasons, many processes employ a biphasic culture process for recombinant protein production (Moore et al. 1997; Kaufmann et al. 1999; Yoon et al. 2003b; Schatz et al. 2003; Fox et al. 2004; Fogolin et al. 2004, Bollati-Fogolin et al. 2005; Trummer et al. 2006). This culture process consists of two phases: an initial proliferation phase designed to maximize biomass at 37°C, followed by an extended production phase, at reduced temperature (28–33°C), during which, the cells remain growth arrested while the production of heterologous proteins is maintained at a high and stable level. More examples of increased productivity achieved through temperature shift are covered in Table 1.

Currently there is very little known regarding the mammalian cell response to reduced culture temperature at the molecular level. It has been proven that it is an active response and cells synthesize specific cold inducible proteins (Kondo et al. 1992; Holland et al. 1993; Jones and Inouye 1994; Derry et al. 1995; Nishiyama et al. 1997a, b; Kaufmann et al. 1999; Danno et al. 2000; Sonna et al. 2002; Dresios et al. 2005; Yoon et al. 2006; Baik et al. 2006; Al-Fageeh et al. 2006). A change in the phosphorylation patterns at the tyrosine residues of two proteins (~180 and 80 kDa) was also observed following reduction of the culture temperature providing further proof that there is an active signalling response to mild hypothermia in CHO (Kaufmann et al. 1999). The effect of reduced temperature on cells is likely to be multifactorial as it also combines the effect of changes in oxygen concentration due to the higher dissolved oxygen concentrations at reduced temperatures (Ohsaka et al. 2002).

Although global protein translation is reduced at lower temperatures in mammalian cells (Wassmann et al. 1998; Phadtare et al. 1999), more than 20 cold inducible proteins have been identified, including cold-inducible RNA binding protein (CIRP, also known as hnRNP A18), RNA binding motif protein 3 (RBM3), protein disulphide isomerase (PDI), NSR1, vimentin, NDK B, ERp57, gi-27754065, phosphoglycerate kinase, heat shock cognate 71 kDa protein, HSP90-β and EF2, (Nishiyama et al. 1997a, b; Sheikh et al. 1997; Kondo et al. 1992; Danno et al. 2000; Sonna et al. 2002; Al-Fageeh et al. 2006; Baik et al. 2006). In this laboratory global proteomic analysis of cells during exponential phase growth at 37°C and low temperature induced stationary phase at 31°C has resulted in the identification of 201 differentially expressed proteins out of 2,852 detected, of which 118 were upregulated (Kumar et al. in preparation).

Two cold shock proteins, CIRP and RBM3, have been studied extensively. Both CIRP and RBM3 are involved in modulation of transcription and translation by functioning as RNA chaperones (Nishiyama et al. 1997a, b; Danno et al. 2000; Sonna et al. 2002; Dresios et al. 2005). These both proteins are highly similar and belong to the glycine rich RNA-binding protein family that are characterized by a consensus sequence RNA-binding domain (CS-RBD) at the N-terminus and a glycine-rich domain at the C-terminus (Derry et al. 1995). CIRP was the first protein identified that was induced following reduction of temperature in mammalian cells (Nishiyama et al. 1997a, b). Apart from CHO, CIRP has been detected in mouse, rat, and human cells, and its sequence is highly conserved in these species (Nishiyama et al. 1997a, b; Xue et al. 1999). The

expression of CIRP has also been observed increasing at exposures to other stress conditions, such as ultraviolet radiation or hypoxia, although heat stress dose not affect CIRP levels (Nishiyama et al. 1997a, b; Fujita et al. 1999; Yang and Carrier 2001). Inhibition of CIRP expression using antisense oligonucleotides allowed the reversal of cold induced growth arrest indicating that CIRP is at least a component of the low temperature induced growth suppression (Nishiyama et al. 1997b). CIRP also specifically binds to the 3′ untranslated region (UTR) of susceptible RNAs protecting and restoring their native confirmations during stressful conditions and this binding has been shown to improve both the efficiency of translation and RNA stability (Yang and Carrier 2001). In recent studies, 4-fold increases were observed in CIRP expression in both EPO and FSH expressing CHO at 32°C in comparison of standard 37°C culture (Yoon et al. 2006). No significant permanent change (decrease or increase) in CIRP expression was noticed through further adaptation of both cell lines to reduced temperature, which may be because the effect of cold shock was eliminated during the adaptation process. The growth rates of reduced-temperature-adapted CHO were improved by 73 and 20%, respectively, despite static CIRP levels. It is clear therefore that other factors are involved in the control of cell growth at reduced temperature (Yoon et al. 2006).

RBM3 is another well-studied protein that is induced in mammalian cells at reduced temperature. The RBM3 gene resides on the Xp11.2 region in humans and codes for a protein very close in structure to other human RNA-binding proteins and shares 94% identity in amino acid sequence with mouse RBM3 (Derry et al. 1995; Danno et al. 2000). RBM3 is increased at reduced temperature in various cells, i.e. HepG2, NC65, HeLa, T24, K562 and TAMA26 (Danno et al. 2000) and has been identified in CHO also (Hayduk et al. 2004). RBM3 has been found to be involved in cytokine-dependent proliferation (Baghdoyan et al. 2000), poxvirus replication (Wright et al. 2001), development of cancer (Baldi et al. 2003) and apoptosis (Kita et al. 2002). Expression of RBM3 at reduced temperature was shown to result in higher relative levels of 80S monosomes and polysomes consistent with an increased association of ribosomal subunits during the initiation step of protein synthesis (Dresios et al. 2005). A tight physical association of RBM3 with 60S ribosomal subunits that did not require RNA or nascent polypeptide chains has also been reported. Several deletion and mutational analysis experiments on a putative IRES within the 720 bp 5′ leader sequence of the RBM3 mRNA revealed at least nine discrete *cis*-acting sequences, including a 22-nt IRES module (a 10-nt enhancer and two inhibitory sequences), and four of them probably bind specifically to distinct cytoplasmic proteins (Chappell and Mauro 2003). As a result of these findings, it is thought that the 5′-UTR of RBM3 mRNA contains a number of specialized sequences that facilitate cap-independent translation to ensure mRNA translation upon cold shock, despite general (cap-dependent) mRNA translation being compromised (Chappell et al. 2001; Chappell and Mauro 2003). Decreased levels of microRNAs were also reported in mouse N2a neuroblastoma cells expressing an RBM3-c-Myc fusion protein, indicating that RBM3 can interact with the miRNA fraction and hence regulate global levels of protein synthesis (Dresios et al. 2005). RBM3 like CIRP can be regulated by changes in oxygen levels which further indicates a link between the low-temperature and oxygen-response pathways (Wellmann et al. 2004).

PDI protein also seems to respond to reduced temperature as its expression was reported as being almost 7-fold increased following reduction of the culture temperature to 33°C (Baik et al. 2006). This may explain some of the effects of cold shock on productivity as PDI functions as endoplasmic reticulum chaperone through participating in the folding of proteins containing disulfide bonds, PDI has also been linked to increased secretion of heterologous proteins (Robinson et al. 1994; Ostermeier et al. 1996; Shusta et al. 1998). PDI levels were almost 2-fold higher in NS0 cells associated with high specific productivity of MAb (Smales et al. 2004; Alete et al. 2005). The effects of PDI on productivity may be cell line- and product-specific, but as overexpression of PDI in CHO was also shown to decrease the secretion of a disulfide-rich tumour-necrosis-factor-receptor

(TNFR):Fc fusion protein (Davis et al. 2000), it is possible that effects of PDI on productivity may be dependent on other factors such as BiP or ENPL (Smales et al. 2004).

Therefore although the effects of low culture temperature on the cellular productivity of recombinant proteins are variable and depend on the temperature, cell line, clone, expression system and/or protein, the effect of reducing cell growth is a universal phenomenon (Furukawa and Ohsuye 1998, 1999; Kaufmann et al. 1999; Ryll et al. 2000; Schatz et al. 2003; Yoon et al. 2003a, b, 2004, 2006; Fox et al. 2004; Fogolin et al. 2004, Bollati-Fogolin et al. 2005; Trummer et al. 2006; Al-Fageeh et al. 2006).

(II) Cell engineering based approaches

Currently most cell engineering based approaches to control proliferation and enhance productivity are at the proof of concept stage and do not form a major part of most industrial production runs. These studies have allowed researchers to specifically identify which molecules are important for regulation of proliferation.

Cyclin dependent kinases (CDKs) are key components of the pathways that control cell cycle transition (Hengst et al. 1994; Sugimoto et al. 2002) and therefore are an important target for achieving control of cell proliferation. Cyclins, phosphorylation and the formation of ternary complexes with cyclin dependent kinase inhibitors (CKI), such as $p21^{CIP1}$ and $p27^{KIP1}$, direct CDK activity (Grana and Reddy 1995). Synthesis and destruction of cyclins or phosphorylation of CKI complexes ensures an appropriate activation of their CDK partners and therefore maintains the balance and directionality of the cell cycle (Hayles et al. 1994; Nurse 1994). $p21^{CIP1}$ is induced by the tumour suppressor gene p53 in response to genotoxic stress as part of the cellular program to repair DNA damage through interaction with the replication and repair factor, proliferating nuclear antigen (PCNA) or in response to ribonucleotide depletion to allow cells to replenish metabolic precursors prior to a subsequent round of DNA replication (Mazur et al. 1998). $p21^{CIP1}$ expression is also induced by tumor growth factor β (TGF-β) directly resulting in p53 independent cell-cycle arrest (Reynisdottir et al. 1995). At elevated intracellular concentrations $p21^{CIP1}$ binds to cyclin-CDK complexes and inhibits the progression of cell cycle at the G1/S transition phase (Bi et al. 2004).

$p21^{CIP1}$-arrested CHO are approximately 4-fold bigger than proliferating CHO, are metabolically more active and demonstrate a corresponding increase in protein synthesis (Bi et al. 2004). Similarly specific productivity increased 4-fold in NS0 cells expressing a chimeric IgG4 antibody following $p21^{CIP1}$ arrested NS0 cells (Watanabe et al. 2002; Ibarra et al. 2003).

$p27^{KIP1}$ exerts G1-cell cycle arrest by binding to cyclin/CDK complexes, i.e. cyclin E/CDK2, Cyclin A/CDK2 and cyclinD/CDK4 all of which are required for transition of cells from G1 to S phase (Stein et al. 1999; Toyoshima and Hunter 1994). TGF-β treated Mv1Lu mink lung epithelial cells and human keratinocytes were reported to have increased $p27^{KIP1}$ levels resulting in cell cycle arrest (Reynisdottir et al. 1995; Sgambato et al. 2000). The use of $p27^{KIP1}$ under control of the Tet-switch (tetracycline-responsive promoter ph^{CMV-1}) facilitated a growth-arrested extended production phase of at least 7 days and enhanced volumetric SEAP production in CHO (Mazur et al. 1999), other data however links this increase in specific productivity following $p27^{KIP1}$ arrest to a higher expenditure of cell energy as demonstrated by nutrient consumption (oxygen, glutamine and glucose) and waste production (lactate and ammonia) (Carvalhal et al. 2003).

p53, as well as promoting $p21^{CIP1}$, also induces other effectors of cell-cycle arrest such as GADD45 and the insulin-like growth factor binding protein 3 (IGF-BP3), all of which exert a cell-cycle block at G1-phase (El-Deiry et al. 1994; Buckbinder et al. 1995; Rowan et al. 1996; Mazur et al. 1998) making p53 one of the most important growth suppressors in eukaryotes. However it also induces apoptosis in some cell lines (El-Deiry et al. 1993; Ko and Prives 1996). A p53 mutant, p53175P, has been generated which causes cell-cycle arrest but not apoptosis in primary rat embryo fibroblasts (Rowan et al. 1996) and has been used to study increased productivity in cell cycle arrested CHO

Table 2 Effect of cell engineering on cell cycle and productivity

Contributor	Cell line and recombinant product	Effectors	Specific productivity (Qp) (fold increase)
Fussenegger et al. (1997)	CHO SEAP	$p21^{CIP1}$ or $p27^{KIP1}$ or p53175P	10
Fussenegger et al. (1998)	CHO SEAP	$p21^{CIP1}$	10–15
Mazur et al. (1998)	CHO SEAP	$p21^{CIP1}$, $p27^{KIP1}$ or p53175P	10–15
Fussenegger et al. (1998)	CHO SEAP	$p27^{KIP1}$ + Bcl-2	30
Kaufmann et al. (2001)	CHO SEAP	$p27^{KIP1}$ and reduced temperature culture (30°C)	2–3
Watanabe et al. (2002)	NS0 human-mouse chimeric IgG4 antibody	$p21^{CIP1}$	>4
Meents et al. (2002)	CHO soluble intercellular adhesion molecule (sICAM)	$p27^{KIP1}$	5
Ibarra et al. (2003)	NS0 chimeric antibody	$p21^{CIP1}$	4
Carvalhal et al. (2003)	CHO SEAP	$p27^{KIP1}$	4
Bi et al. (2004)	CHO human-mouse chimeric IgG4 antibody	$p21^{CIP1}$	~4

(Table 2) (Mazur et al. 1998; Fussenegger et al. 1997). In experiments to compare the effects of growth inhibitors, the increase in cell specific productivity following expression of p53175P, $p21^{CIP1}$ or $p27^{KIP1}$ are comparable to each other, the improvement in production therefore being the effect of cell cycle arrest rather than due to any specific growth inhibitor (Fussenegger et al. 1997).

Apart from CDKs and CKIs, expression of Bcl-2 can also cause cell cycle arrest in G1-phase of murine hybridoma cells (O'Reilly et al. 1996; Simpson et al. 1997, 1999). About 80% of hybridoma cells expressing Bcl-2 accumulated in G1 phase of the cell cycle (Simpson et al. 1999). The duration of G1-phase was also increased by 75% due to Bcl-2 expression in NS0 cells, although cell cycle arrest occurred in the G2 phase (Tey and Al-Rubeai 2005). Bcl-2 is known for its anti-apoptotic activity, however its cell cycle modulation activity has been reported to be distinct from its anti-apoptotic domain (Huang et al. 1997).

The comparison of cell cycle arrest through reduced culture temperature or heterologous gene expression has revealed that temperature shift is currently superior and it is also a more practical approach, direct comparisons include the situation where p27-mediated growth arrest greatly enhances specific productivity (~15-fold) while low temperature ensures complete and sustained growth arrest leading to a higher overall volumetric productivity (Mazur et al. 1998; Kaufmann et al. 2001). In studies where the expression of p27 was carried out at low temperature, it was found that the temperature effect was dominant (Kaufmann et al. 2001). Arresting cells through reduction of the temperature also has the advantage that all cells are similarly affected whereas engineering approaches allow for the possibility of clones which escape from growth suppression (Mazur et al. 1998). Mutation or loss of the genes necessary for proliferation control may render the situation worse and could provide a substantial growth advantage to mutant cells which leading to overgrowth of the arrested cells and subsequent loss in product yield (Fussenegger et al. 1997, 1998; Kaufmann et al. 2001). Therefore use of temperature shift for recombinant protein production is the method of choice employed in industrial processes.

(III) Chemical approaches for cell cycle arrest

Sodium butyrate (NaBu), a sodium salt of butyric acid, has been used in rCHO cell cultures to improve productivity of recombinant proteins (Chang et al. 1999; Hendrick et al. 2001; Hunt et al. 2002; Kim and Lee 2001, 2002; Lee and Lee 2003). It exerts a wide variety of morphological and functional effects on mammalian cells in culture. NaBu can cause

cell blockage at the G1-phase of the cell cycle (Hendrick et al. 2001; Kim and Lee 2002), inhibit histone deacetylases (Buckley et al. 1996; Pikaart et al. 1998; Cherlet and Marc 2000; Monneret 2005), induce cell differentiation (Garcia-Bermejo et al. 1997) and apoptosis (Kim and Lee 2002). Two CDKs, cdc6 and cdk1, were down regulated in NaBu-mediated cell cycle arrested bovine kidney epithelial cells (MDBK) (Li and Elsasser 2005). Cdc6 has been shown to be rate limiting for initiation of DNA replication in a number of eukaryotic cell lines and is essential for the assembly of the pre-replication complex (Sun et al. 2002) while cdk1 helps in mitosis (Li and Elsasser 2005). Approximately 67% of HeLa cells and 54% of CHO accumulated in the G1-phase following NaBu treatment (Xue and Rao 1981; Hendrick et al. 2001). The specific and volumetric productivity were also increased (Palermo et al. 1992; Hendrick et al. 2001; Kim and Lee 2001, 2002; Lee and Lee 2003). However increased apoptosis was also observed in these cultures therefore undermining the beneficiary effects of improved productivity (Xue and Rao 1981; Chang et al. 1999; Kim and Lee 2001, 2002). To prevent this, anti-apoptotic factors e.g. Bcl-2, antisense molecules for caspase-3, and the proteasome inhibitor MG-132, have been used in CHO in the presence of NaBu resulting in prolonged G1-phase and improved productivity in comparison to NaBu alone (Kim and Lee 2001, 2002; Li and Elsasser 2005).

Pentanoic acid has been reported as an alternate to NaBu as it has comparable enhancing effects on protein production with lower associated growth suppression and apoptosis compared with NaBu (Liu et al. 2001). It was observed that the conformation of the carbon carrying carboxyl group in pentanoate has some role in pentanoate induced growth suppression (Liu et al. 2001). Although the molecular mechanism underlying the stimulation of protein expression and growth suppression effects by pentanoate is not clear, butyrate and pentanoate are expected to have similar mechanism as they share similar carboxyl carbon conformation, inhibit histone deacetylase, and suppress cell growth (Liu et al. 2001).

Dimethyl sulfoxide (DMSO) is a well-known differentiation inducer and has been shown to induce an efficient and reversible G1 phase arrest in CHO cells (Fiore et al. 2002), human lymphoid cell lines (Sawai et al. 1990) and mouse fibroblasts (Srinivas et al. 1991). DMSO-induced growth arrest is associated with restored contact inhibition of cell growth and prevention of density-dependent-apoptosis (Fiore and Degrassi 1999). Addition of 1–2% DMSO to cultures efficiently and reversibly arrested up to 90% of CHO cells (Fiore and Degrassi 1999; Fiore et al. 2002) and 85% of hybridoma cells in G1-phase (Ponzio et al. 1998). DMSO inhibits cell growth by affecting CDK and or CKI activity in cells (Ponzio et al. 1998; Fiore and Degrassi 1999; Fiore et al. 2002). In hybridoma cells, DMSO inhibited cyclin D2/CDK4 and cyclin E/CDK2 kinases due to the combined effect of the down-regulation of cyclin D2, the redistribution of $p27^{KIP1}$ from CDK4 to CDK2 complexes and the induction of $p21^{CIP1}$ (Ponzio et al. 1998). Addition of 1.5% DMSO to adherent CHO cells was shown to result in increased $p27^{KIP1}$ and Bcl-2 expression, resulting in growth arrest, improved cell-adhesion and reduced apoptosis (Fiore and Degrassi 1999). Higher concentrations of DMSO (30%) causedmutagenic effects however (Hakura et al. 1993). No cytotoxic or genetic damage was observed on G1-released CHO cells with following treatment with low concentrations of DMSO (Fiore and Degrassi 1999; Fiore et al. 2002).

Quinidine and thymidine, both cell cycle inhibitors, have also been used for regulating the cell cycle distribution of CHO cells (Al-Rubeai et al. 1992; Andersen et al. 2000). Quinidine and thymidine inhibit potassium channels in cells (Al-Rubeai et al. 1992; Wang et al. 1998; Andersen et al. 2000). The level of potassium channel activity has been shown to be different in proliferating and quiescent stage of cell cycle and reduced potassium channel activity is hypothesized to result in cell cycle arrest (Wang et al. 1998). Following the quinidine treatment, 67% of tPA producing CHO were arrested in G1-phase of cell cycle, however 57% cells were arrested in S phase after thymidine treatment (Andersen et al. 2000).

Media formulation approaches for nutrient based cell proliferation control

Besides fulfilling the nutritional requirements of cells, media formulation and its utilization have been implemented as strategies for regulation of cell proliferation and recombinant protein production. The compounds used in medium as carbon/energy sources are typically glucose and glutamine. Inefficient use (higher uptake than required for normal cell growth) of glucose and glutamine leads to rapid biomass generation, nutrient depletion and by-product accumulation (lactate and ammonium ions, etc.) which often have inhibitory effects on culture longevity and productivity (Ozturk et al. 1992; Zeng et al. 1998; Zeng and Deckwer 1999). Finding alternative compounds that can be used efficiently as carbon and energy sources with lower uptake rates is important because slow consumption of nutrients can lead to reduced/arrested cell growth (Altamirano et al. 2001b). These changes are associated with improved culture longevity and overall productivity.

HeLa cells grow with similar efficiency on galactose or fructose as the main sugar source in the medium instead of glucose (Reitzer et al. 1979). The replacement of glutamine with asparagine for the culture of CHO cells improves both cell growth and productivity (Kurano et al. 1990). The substitution of glucose with galactose (a slow metabolising sugar) and glutamine with glutamate (slow transportation rate in to cell and has only one amino group) reduced the growth rate and improved culture longevity in batch cultures of CHO cells (Altamirano et al. 2000). The reduced waste production (such as lactate and ammonium) and osmolality, due to minimal nutrient consumption, also contributed to this (Altamirano et al. 2000). The regulation of the glucose feed rate in culture also increased culture longevity and t-PA productivity in CHO without affecting cellular specific productivity suggesting that the combination of carbon and energy source availability and growth rate can be a determinant of t-PA production in continuous culture (Altamirano et al. 2001a). This can be used as an alternative application of the biphasic culture method using nutrient shift (Altamirano et al. 2001a, b). The use of a nutritional shift from glucose during the growth phase to galactose for production phase after the third day of batch culture and 12th day of perfusion culture resulted in slow growth rate with enhanced viability and increased t-PA production when applied to CHO (Altamirano et al. 2001b). Replacement of glutamine by glutamate and alternatively changing the carbon source from glucose to galactose in the production phase also resulted in an increase in culture longevity, viability and 1.3-fold improved volumetric t-PA production (Altamirano et al. 2004). This improvement in volumetric productivity was not due to any changes in specific productivity (Altamirano et al. 2004). Recently improved growth and t-PA productivity has been achieved by growing CHO cells in the presence of glutamate (6 mM) with 5 mM glucose and 20 mM galactose compared to a standard culture containing 6 mM glutamate and 20 mM glucose (Altamirano et al. 2006). CHO initially consumed the glucose and then they continued growing on galactose in a second phase with the simultaneous consumption of the endogenous lactate and this resulted in improved culture longevity and reduced waste accumulation (Altamirano et al. 2006).

Conclusion

The increasing demand for therapeutic protein products every year is the main driving force to improve current production systems. Regulation of the cell cycle and hence proliferation has been shown to result in improved cell characteristics and production in CHO systems. Comparing temperature, cell engineering, chemical and nutritional approaches, growth regulation through low temperature cultivation has been proven to be more reliable and efficient. Reduced temperature cultivation improves cell specific as well as overall productivity while the other approaches, have variable outcomes. Although the effects of reduced temperature on productivity can be impressive, it should be noted that it still varies among clones, cell lines and recombinant protein products and is still poorly understood; however, ongoing research in this and other laboratories are trying to understand the effects of cold on cells with the aim of further refining CHO culture processes.

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