Modeling the coupled extracellular and intracellular environments in mammalian cell culture

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ABSTRACT

The regulation of metabolism in mammalian cell culture is closely linked to the process of apoptosis—programmed cell death. Apoptosis negatively impacts culture viability, product yield, and quality. An improved understanding of the interaction between apoptosis and metabolism will give rise to better control over the culture process, and thus improvements in product yield. This study presents a mathematical model that describes both the metabolic fluxes involving the extracellular metabolites and the progression of apoptosis in terms of intracellular caspases, and thus highlights the interactions between these two processes. The model is trained and validated against experimental observations of Chinese Hamster Ovary cell culture producing monoclonal antibody. Importantly, the model describes the continued production of monoclonal antibody in post exponential phase by incorporating different rates of antibody production for separate sub-populations within the culture. A parameter estimability test was applied on the combined model to assess the confidence in parameter estimates.

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1. Introduction

Monoclonal antibodies (mAb), typically produced from mammalian cell cultures, are in increasing demand due to their range of applications in therapeutics, diagnostics, clinical uses, and purification of biomolecules. A major challenge when working with mammalian cell culture is the enhancement of productivity. Product yields are generally lower than those obtained when using microbial host expression system; production costs, conversely, are usually higher. Increased productivity can be attained by improving cell density and extending cell viability. However, the achievement of high cell density in bioreactors is greatly hampered by cell death mechanisms which result in reduced cell density, viability and final mAb titer. One such mechanism, apoptosis, is a major form of cell death in mammalian cell culture (Franek and Dolnikova, 1991; Golstein et al., 1991; Goswami et al., 1999). It is triggered mainly by metabolism-related stressors such as nutrient depletion (nutritional stress) and high levels of toxic metabolites (toxic stress). The links between metabolism and apoptosis have been discussed previously (Majors et al., 2007).

Genetic manipulation provides one approach to addressing cell death, and thus optimizing yield. However, for a given host cell, maximizing cell density in bioreactors depends primarily on manipulating medium conditions to regulate cell metabolism and to reduce death by apoptosis.

Apoptosis is a highly controlled process characterized by the activation of proteolytic caspases (cysteine-dependent aspartate-specific proteases) which, in turn, are activated by various apoptosis-inducing signals. Apoptosis is primarily triggered by two pathways, called the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is triggered when an external cell ligand (e.g. FASL, TNF-αL) binds to a cognate cell receptor. This signals the activation of an initiator, caspase-8, which then activates the executioner, caspase-3 (Aggarwal, 2003; Ashkenazi and Dixit, 1998; Schulze-Osthoff et al., 1998). The intrinsic pathway, in contrast, is triggered by mitochondrial stress that causes the release of cytochrome-c into the cytoplasm, where it combines with Apaf-1 (apoptotic protease activating factor), forming the apoptosome complex. This complex then recruits and activates an initiator, caspase-9, which subsequently activates caspase-3, culminating in apoptosis (Li et al., 1997; Pan et al., 1998; Salvesen and Renatus, 2002; Srinivasula et al., 1998; Zou et al., 1999). The executioner caspase-3 cleaves a range of protein species resulting in characteristic cellular destruction. These two activation

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pathways are not strictly independent. It has been shown that in some cell types active caspase-8 cleaves the pro-apoptotic protein Bid, triggering mitochondrial stress and leading to activation of caspase-9 by the intrinsic pathway (Zha et al., 2000). It has also been reported that activation of caspase-8 can be induced by caspase-9 activity (Slee et al., 1999; Viswanath et al., 2001).

The aforementioned apoptosis pathways can be triggered by a number of factors. Cell-to-cell signaling has been found responsible for triggering the extrinsic pathway of apoptosis in response to high cell density or low nutrient levels (Nagata and Golstein, 1995; Yang et al., 2008). The intrinsic pathway has been shown to be activated by nutritional stress resulting from deprivation of essential nutrients, such as glucose (Goswami et al., 1999) and depletion of amino acids, such as glutamine and asparagine (Sanfeliu and Stephanopoulos, 1999; Simpson et al., 1998). Other factors that can trigger the intrinsic pathway include accumulation of toxic byproducts, such as ammonia and lactate (Lao and Toth, 1997; Singh et al., 1994), high osmolality (Takagi et al., 2000), hypoxia, hyperoxia (Barazzone and White, 2000; Petrache et al., 1999; Shoshani et al., 2002), low pH (Osman et al., 2001, 2002), and serum withdrawal (Even et al., 2006).

Mathematical modeling provides a means to better understand process behavior. Earlier metabolic models of mammalian cell culture have generally focused, for simplicity, on balancing the concentrations of nutrients and by-products in the extracellular environment. To that purpose, several metabolic flux models have been developed for various cell culture types (Bailey, 1991; Bonarius et al., 1996; Dorka et al., 2009; Follstad et al., 1999; Gambhir et al., 2003; Gao et al., 2007; Sengupta et al., 2011). These models did not explicit describe the stages of cell death, and thus ignored a range of cell phenotypes that occur during the culture. In these models, cell death rate is generally correlated to the depletion of primary nutrients (e.g. glucose and glutamine) or to accumulation of inhibitory products (e.g. ammonia and lactate). However, cell death can occur well before the depletion of glucose or glutamine, and when levels of inhibitory metabolites are low, suggesting that in some cases there are additional factors involved in cell death (Ljunggren and Haggstrom, 1994). Because they did not distinguish stages of cell death, these previously reported models assigned a single mAb production rate to the entire population of viable cells. However, in our previous studies of hybridoma cell culture, we found mAb production continued into the post-exponential phase, at which time the culture was largely apoptotic (Dorka et al., 2009). This observation suggests that apoptotic cells continue to produce mAb, possibly at a different rate than normal healthy cells. Consequently, an accurate model of mAb production should describe the progression of apoptosis in the culture and account for mAb production from apoptotic cells.

Our earlier efforts generated a population-balance model of caspase activity in cell culture (Meshram et al., 2012). That model incorporated fixed input profiles of nutrient and toxin concentrations; metabolism was not specifically modeled. While that model provides a valuable description of the progression of apoptosis, it is not sufficient for direct enhancement of process yield. Optimization involves manipulating the extracellular environment to maximize productivity, and so demands a model that describes the dynamic evolution of this extracellular environment. This paper presents a model that incorporates both the extracellular metabolites and the intracellular caspase species, and thus provides a dynamic description of the evolution of apoptosis and the coupling between the extracellular and intracellular environments. Measurements of caspases obtained by flow cytometry were combined with data on metabolites to calibrate the model and test its predictive accuracy. This comprehensive model provides an improved description of the cell culture process and will serve as a foundation for future strategies to enhance cell density and product titer.

2. Materials and methods

2.1. Cell culture

A Chinese hamster ovary (CHO) cell line producing Anti-RhD (Cangene Corporation, Mississauga, Canada) was used. Cell culture was initiated in T-flasks and scaled-up to 500 ml spinner flasks in batch mode. The culture was grown in HyClone SFX-CHO medium and supplemented with glutamine (Sigma Aldrich Ltd.) and fetal bovine serum (Invitrogen). The culture in suspension in spinner flasks was agitated at 100 RPM in a humidified CO_2 incubator (Sanyo IR Sensor, 37 °C, 5% CO_2).

2.2. Cell count and viability

Cell density and viability were determined by microscopic counting with a hemocytometer using the conventional trypan blue dye exclusion method (Phillips, 1973).

2.3. Glucose, lactate, amino acids, and ammonia assay

Glucose was measured enzymatically using a glucose test kit (Megazyme International, Bray, Ireland). Lactate measurements were performed enzymatically using a lactate assay kit (Eton Bioscience, San Diego, USA). A high-performance reverse-phase chromatography (HPLC) method using the pre-column derivatization technique with phenylisothiocyanate (PITC) was employed for the quantification of amino acids. Ammonia was assayed using a pH/ISE meter equipped with an Ammonia Ion-Selective electrode (VWR, model 710A).

2.4. Caspase activity assays

Caspase activity was assayed by flow cytometry using the fluorophore-bound caspase inhibitor (FLICA) method, targeting specific activated caspases (Meshram et al., 2012). Four caspase assays were performed: a green (carboxyfluorescein) FLICA caspase-8 assay, a green FLICA caspase-9 assay, a red (sulforhodamine) FLICA Caspase-3 & 7 assay, and a red (sulforhodamine) FLICA caspase-9 assay (ImmunoChemistry Technologies, LLC). (Caspase-7 was not considered in this study.) Flow cytometry analysis was started approximately 1 day after seeding, at which time the cultures had entered exponential phase and exhibited a viability of approximately 99%. For each culture, three aliquots were collected at each time point. One was tested for levels of caspase-8 and caspase-3 activation, the second for activated caspase-9 and caspase-3 and the third for caspase-8 and caspase-9 activation. (Simultaneous testing of all three caspases would have been preferred; however, no such three channel assay is currently commercially available.) The fluorescence of 25,000 cells (events) per sample was acquired on a flow cytometer (Becton Dickinson FACS Vantage SE). The resulting flow cytometry data were analysed by WinMDI v2.9 (Scripps Research Institute, CA, USA). Cells were classified based on their caspase activation status by setting intensity thresholds for each caspase; negative controls were taken to be the measured levels at day one, when apoptotic cell numbers are very small. For each test, a dot-plot was divided into four quadrants thus categorizing cells into four sub-populations, resulting in a total of twelve caspase level combinations: $C8^-C3^-$, $C8^+C3^-$, $C8^+C3^+$, $C8^-C3^+$, $C9^-C3^-$, $C9^+C3^-$, $C9^+C3^+$, $C9^-C3^+$, $C8^-C9^-$, $C8^+C9^-$, $C8^+C9^+$, $C8^-C9^+$. (The positive or negative subscripts refer to cells testing either positive or negative for the corresponding active caspase species.) On day one the culture showed minimal caspase activity. Therefore, the flow cytometry plots were used as negative controls so as for each of the three caspases populations, a threshold was selected that established an