Abstract: Caspases are the key mediators of the apoptotic cascade leading to cell death in mammalian cells. Caspase activation in a Chinese Hamster Ovary (CHO) cell line producing Anti-RhD monoclonal antibody (mAb) in batch culture was simulated and measured experimentally. The model assumes that apoptosis proceeds through one of two independent pathways: the intrinsic pathway and the extrinsic pathway. Induction of the extrinsic pathway is assumed to involve cell-cell signaling by apoptotic (caspase-3 active) cells, while the intrinsic pathway is assumed to be triggered by toxic stress (ammonia). The model parameters were estimated by training the model by a nonlinear constrained optimization method embedded in MATLAB. The model provides a good fit to the experimental observations. In addition, this apoptosis model was integrated with a dynamic model of CHO culture metabolism previously obtained through metabolic flux analysis. The combined model provides good predictions of the measured metabolite profiles thus it can serve for future optimization of the culture.

Keywords: CHO, Apoptosis, Caspases, Modeling, Metabolic flux

1. INTRODUCTION

Mammalian cell culture is the most predominantly used expression system for the commercial production of biopharmaceuticals that require strict post-translational modifications, such as competent glycosylation, for full biological function. Among these products, monoclonal antibodies, mAbs have found a variety of applications on medical, academic and commercial levels.

A key challenge in working with mammalian cell culture is the enhancement of productivity since product yield is usually significantly lower than when using microbial host expression systems. The enhancement of productivity is required to meet high market demand. However, improving productivity has been a challenging task because of these cultures complex cellular machinery. All cell culture systems involve a range of intracellular and extracellular metabolites interlinked through different metabolic pathways, and this interconnection varies with the type of cell line. Once critical nutrients fall short for full metabolic activity of the culture, cell growth stops and the cells may enter apoptosis, a process of programmed cell death. Apoptosis is the major form of cell death in bioreactors (Golstein et al., 1991; Goswami et al., 1999) and thus negatively impacts cell density, cell viability and consequently product titre. Process operation in fed-batch mode or perfusion can enhance cell density, and thus productivity, by delaying the onset of apoptosis.

Apoptosis is a highly controlled, complex phenomenon and may be triggered by two major pathways: the extrinsic pathway and the intrinsic pathway. These two pathways are activated by caspase-8 and caspase-9, respectively. These so-called initiator caspases each subsequently activate caspase-3, the executioner caspase. The extrinsic pathway is triggered when an external cell ligand binds to cognate cell receptors of another cell. This signals the activation of caspase-8 which further activates caspase-3 (Ashkenazi and Dixit, 1998). The intrinsic pathway is triggered by any mitochondrial stress that causes the release of cytochrome-c into the cytoplasm resulting in activation of caspase-9, and subsequent activation of caspase-3 (Li et al., 1997; Salvesen and Renatus, 2002; Srinivasula et al., 1998). The executioner caspase-3 is responsible for the cleavage of survival protein resulting in destruction of cells, which is the characteristic of apoptosis. Cross-talk between these pathways has been observed in certain cell types when caspase-8 activates the intrinsic pathway. In these cases active caspase-8 cleaves the proapoptotic protein, Bid, which triggers mitochondrial stress, leading to activation of caspase-9 by the intrinsic pathway (Li et al., 1998).

There have been various triggers observed in mammalian cell culture that can give rise to apoptosis. Cell-cell signalling can activate the extrinsic pathway in response to cell density, nutrient levels, or the presence of toxic metabolites. The intrinsic pathway has been found to be triggered at the event of deprivation of essential nutrients such as glucose (Goswami et al., 1999), and amino acids, such as glutamine.
or asparagine (Sanfeliu and Stephanopoulos, 1999; Simpson et al., 1998). Also, accumulation of toxic products, such as ammonia and lactate, high osmolarity, hypoxia, and hyperoxia have been found responsible for inducing this pathway of apoptosis (Lao and Toth, 1997; Singh et al., 1994). Deprivation of serum has also been found to stop growth and to trigger this apoptotic pathway (Even et al., 2006). Serum is an undefined supplement commonly needed for cell growth. It contains several unknown essential components such as vitamins, hormones, growth factors, and cytokines. This uncertainty presents a problem in evaluating which of these components in serum should actually be driving cell growth, other than glucose and glutamine which are generally considered essential growth.

Modelling the cell culture processes that interlink metabolism and apoptosis would give a better understanding of apoptosis and help in devising strategies for delaying its onset. In our previous studies, it was found that apoptotic cell culture continues producing mAbs, but does so at different rates than normal healthy culture. The current study proposes a comprehensive dynamic model for a CHO cell line that describes the progression of apoptotic cell populations (triggered by the two pathways) and the resulting effect on the evolution of extracellular metabolites such as glucose, glutamine and ammonia. To our knowledge this is the first attempt at modelling the direct interrelation between extracellular nutrients and intracellular apoptosis-related events; the model can serve as the basis for optimizing the feeding of nutrients to affect the progression of apoptosis.

2. MATERIALS AND METHODS

Cell line and Medium

Chinese hamster ovary (CHO) cell line IgG-9f8 (obtained from Cangene Corporation, Mississauga) producing Anti-RhD was used in this study. Cell culture was initiated in T-flask and scaled-up to 500ml in spinner flasks in batch mode. HyClone SFX-CHO medium was used to support growth. The medium was supplemented with glutamine (Sigma Aldrich Inc.) and fetal bovine serum (Invitrogen Corp.).

Culture Conditions

Cell suspension in spinner was agitated at 100RPM in a humidified CO2 incubator (Sanyo IR Sensor, 37 °C, 5% CO2)

Experimental Design

Four different batch cultures were prepared in spinner flasks, labelled SP1-4, with initial cell densities at 0.15x10^6, 0.3x10^6, 0.26x10^6 and 0.77x10^6 cells/mL respectively. (The two intermediate concentration values of 0.3x10^6 and 0.26x10^6 cells/mL were designed as replicates but ended up being slightly different due to experimental variability). Spinner flask 1 (SP1), which was seeded with 0.15x10^6 cells/mL, was supplemented with 1% fetal bovine serum and 1mM glutamine. The same initial ratio of glutamine and serum per cell was maintained in the remaining three spinner flasks to avoid premature nutrient depletion in higher-cell-density spinners.

Cell density and viability assay

Cell growth was monitored by regular sampling of cell suspension from spinner and cell counting. Cell density and viability were determined by microscopic counting with a haemocytometer using the trypan blue dye exclusion method.

Ammonia and lactate assay

Ammonia was measured with a pH/ISE meter equipped with an Ammonia Ion-Selective electrode (VWR, model 710A). Lactate assays were performed enzymatically using a lactate assay kit (Eton Biosciences, Inc).

Glucose Assay

Glucose in cell culture samples was assayed enzymatically using a glucose test kit (Megazyme Glucose Test Kit).

Caspase assay

Flow cytometry was performed to verify the presence and to track the progress of caspase activation. FLICA assays that use fluorophore-bound caspase inhibitors that target specific activated caspases were used for this purpose. A green (carboxyfluorescein) FLICA caspase-8 assay kit, a red (sulforhodamine) FLICA Caspase-3 & 7 & kit and a red (sulforhodamine) FLICA caspase-9 assay kit (ImmunoChemistry Technologies, LLC) were used. Flow cytometry analyses began approximately 1 day after seeding at which time the cultures had entered exponential phase. For each spinner flask, three aliquots were collected at each time point. One was tested for levels of caspase-8 and 3 activation, the second was tested for activated caspase-9 and 3 and the third for caspase-8 and caspase-9 activation. (Although the simultaneous testing of all caspases would be preferred, no such assay is currently available commercially). For each sample, the fluorescence of 25,000 cells was measured on a flow cytometer (Becton Dickinson FACS Vantage SE). Analyses of FCM data were performed using WinMDI v2.9 (Scripps Research Institute, CA, USA). To classify cells based on their caspase activation status, intensity thresholds for each caspase were set by using the single dye negative controls prepared at day one when the measurements of caspases started. For each FCM test, cells were then categorized into four subpopulations, resulting in a total of eight cell conditions: C8-, C8+C3-, C8+C3+, C8-C3-, C8-C3+, C9-C3-, C9+C3-, C9+C3+, C8-C9+, C8+C9-, C8+C9, C8-C9, C8-C9+ where ‘+’ indicates activity and ‘-‘ indicates no activity in the respective caspase.

Modeling

To construct a model, the presence of different cell subpopulations based on various caspase activities was hypothesized, namely normal healthy cells (x1), apoptotic cells testing positive for caspase-8 but negative for caspase-3 (x2), apoptotic cells testing positive for caspase-9 but negative for caspase-8 (x3) and apoptotic cells testing positive for caspase-3 (x4). It is proposed that normal healthy cells (x1) undergo apoptosis by either of the two pathways as described above: the extrinsic pathway forming caspase-8 active cells (x2) and the intrinsic pathway forming caspase-9 active cells (x3). Finally these two subpopulations are transformed into
caspase-3 active cells ($x_4$) as shown in the Figure 1. These caspase-3 active cells undergo death to form dead cells ($x_5$). The viable cell population was assumed to be composed of viable dividing/growing cells ($X_g$) and viable non-dividing/non-growing or resting cells ($X_{ng}$) where the latter consume substrate for maintenance but not for growth. Since the actual estimate of resting cells was not available, it was expressed as a fraction of resting cells. The fraction ($f_{gr}$) of dividing or growing cells is described by a Tessier growth model (Moser, 1985) as follows,

$$f_{gr} = 1 - e^{-\frac{x}{K_s}}$$

Where $S$ is the limiting nutrient. Since neither glucose nor glutamine (nor any other nutrient) was found to be limiting in this study, $S$ ($x_5$) was defined as the fraction of an unknown nutrient remaining in the culture. It is suspected that this unknown nutrient is present either in basal medium or in serum.

The growth rate of normal viable healthy cells is expressed as:

$$v_1 = \mu_g f_{gr} x_1$$

Based on observations of the growth rates in cultures of differing densities, the specific growth rate ($\mu_g$) was expressed by a logistic model and given by:

$$\mu_g = \mu_L \left( 1 - \frac{x_1 + x_2 + x_3 + x_4}{X_{max}} \right)$$

The rate of the onset of the extrinsic pathway ($v_2$) is taken to be dependent on the density of caspase-3 apoptotic cells ($x_4$), as a result of cell-to-cell interaction (Hardy and Stark, 2002).

This is given by

$$v_2 = (\mu_e x_4^2) x_1$$

The onset rate of the intrinsic pathway was considered to be triggered via nutritional stress (absence of nutrient $S$) and is given by

$$v_3 = \mu_i \frac{1}{1 + k_{sg} S} x_1$$

Also an independent growth term was added in caspase-8 ($x_2$) and caspase-9 active cells ($x_3$) as follows

$$v_{2g} = \mu_L f_{gr} x_2$$
$$v_{3g} = \mu_L f_{gr} x_3$$

The rates $v_4$ and $v_5$ were kept constant and are given as

$$v_4 = k_{x_4} x_2$$
$$v_5 = k_{x_5} x_3$$

The death rate of caspase-3 active cells ($v_6$) is given as

$$v_6 = \mu_d x_4$$

The specific death rate ($\mu_d$) was assumed to be a nonlinear function of the concentration of ammonia in the culture:

$$\mu_d = \mu_{amm} Amm^\beta$$

The disappearance or lysis rate of the dead cells is given as

$$v_7 = k_{lyx} x_5$$

And the substrate ($S$) consumption rate was expressed as

$$v_8 = -k_{sub} (x_1 + x_2 + x_3)$$

The total viable cells that still have the capability to keep producing mAb were defined as

$$X_v = x_1 + x_2 + x_3 + x_4$$
Table 1 Metabolite model equations

\[
\begin{align*}
\frac{d\text{Glc}}{dt} &= -a_{11}X_g - \left[ \frac{a_2 \text{Glc}}{K_{\text{Glc}+\text{Glc}}} + 0.5 \frac{a_3 \text{Glc}\text{Gln}}{(K_{\text{Glc}+\text{Glc}G}\text{Gln})} \right] X_{ng} \\
\frac{d\text{Lac}}{dt} &= a_{22}X_g + \left[ \frac{2a_2 \text{Glc}}{K_{\text{Glc}+\text{Glc}}} + \frac{a_3 \text{Gln}}{K_{\text{Gln}\text{Gln}}} \right] X_{ng} \\
\frac{d\text{Gln}}{dt} &= -a_{33}X_g - \left[ \frac{a_5 \text{Gln}}{K_{\text{Gln}\text{Gln}}} + \frac{a_7 \text{Gln} \text{Gln}}{(K_{\text{Gln}+\text{Gln}} \text{Gln})} \right] X_{ng} \\
\frac{d\text{Glu}}{dt} &= a_{44}X_g + \left[ -\frac{a_3 \text{Glu}}{K_{\text{Glu}\text{Glu}}} + \frac{a_7 \text{Gln} \text{Gln}}{(K_{\text{Glu}+\text{Glu}} \text{Gln})} \right] X_{ng}
\end{align*}
\]

The model was set up by generating material balance equations and implemented in MATLAB (version 7.10.0.499, R2010a; Mathworks, USA). The final structure of the model presented above was determined based on extensive comparisons of alternative structures in terms of fitting and prediction abilities. Parameter fitting began with the use of a genetic algorithm to obtain approximate values of the optimal model parameters. These parameters values were refined by the application of a constrained nonlinear optimization function (Matlab routine: fmincon).

The experimental data in SP1 and SP4 were used for fitting while the data from SP2 and SP3 was used for validating the model. The model was calibrated by minimizing the relative sum-of-squares errors between the model predictions and the corresponding measured values. The model state variable \(x_i\) was compared to C8-C9, \(x_j\) to C8’C3’, and \(x_k\) to C9’C3’. In the case of \(x_i\), there was generally a small experimental discrepancy between the measured population of caspase-3 active cells in the two populations (corresponding in one case to the sum of C8’C3’ and C8’C3’ and to the sum of C9’C3’ and C9’C3’ in the other). The state \(x_j\) was compared against both values in the fitting algorithm. The model was also inspected for its performance when it was combined with a dynamic metabolites’ model based on flux analysis as per the interconnections shown in Table 1. The variables shared by the two models are \(X_g\) and \(X_{ng}\).

3. RESULTS AND DISCUSSION

The model proposed for caspase activation was dynamically simulated for CHO cell culture in batch mode the lowest and highest initial cell density experiments (SP1 and SP4). Figure 2A-2C and Figure 2D-2F show the resulting fits. Table 2 shows the corresponding model parameters. Using these parameters, the model was validated with the experimental results obtained in SP2 and SP3 which differed in initial cell density. Figure 2G-2I show the prediction of SP2 behavior while Figure 2J-2L show the prediction on SP3 data. It can be seen that the model is able to capture satisfactorily the evolution of caspase subpopulations triggered in the extrinsic and intrinsic pathway. The two experiments SP1 and SP4 differ in initial cell density and show different initial rates of evolution of subpopulations, particularly \(x_i\).

The model displays a slow decrease in unknown substrate \(S\) in SP1 as compared to that in SP4, which leads to the faster build up of caspase-3 active cells in SP4 compared to SP1. The simulated concentration profile captures this behavior, and also shows good agreement with the experimental observations of total viable and dead cells.

Investigation of the model revealed its ability to predict the metabolite profiles with metabolic flux model developed in our group (Naderi et al., 2010). The metabolite model used was obtained by metabolic flux analysis and it was calibrated with data not used for the calibration of the caspases’ model, in order to test its prediction capabilities. It was found that the model predicted the levels of ammonia, lactate, and glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth. It is known to trigger the intrinsic pathway of apoptosis, but the level of glucose in the culture. The fits are shown in Figure 3. Lactate has been found previously to inhibit growth.

Table 2. Parameters involved in model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>(K_f)</td>
<td>0.64</td>
</tr>
<tr>
<td>(\mu_e)</td>
<td>1.34 day(^{-1})</td>
</tr>
<tr>
<td>(X_{max})</td>
<td>1.6 ((10^6)) cells/ml</td>
</tr>
<tr>
<td>(\mu_i)</td>
<td>2.94 (cell/ml)(^{-1}) day(^{-1})</td>
</tr>
<tr>
<td>(k_{X3})</td>
<td>0.33 day(^{-1})</td>
</tr>
<tr>
<td>(k_{X4})</td>
<td>0.31 day(^{-1})</td>
</tr>
<tr>
<td>(\mu_{amm})</td>
<td>0.12 mM(^{-1}) day(^{-1})</td>
</tr>
<tr>
<td>(\alpha)</td>
<td>1.9</td>
</tr>
<tr>
<td>(\beta)</td>
<td>1.2</td>
</tr>
<tr>
<td>(k_{Ips})</td>
<td>0.11 day(^{-1})</td>
</tr>
<tr>
<td>(k_{sub})</td>
<td>0.97 mM(^{-1}) (cell/ml)(^{-1}) day(^{-1})</td>
</tr>
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</table>
Fig. 2: Comparison of experimental observations with simulated profiles. The data points represent the experimental observations and solid curves represent the simulated profiles.

Fitting: A-C: SP1, D-F: SP4; Prediction: G-I: SP2, J-L: SP3

A, D, G, J: (∗) Experimental profile of $x_1$, (—) simulated profile of $x_1$,
(ο) Experimental profile of $x_2$, (—) simulated profile of $x_2$,
B, E, H, K: (∗) Experimental profile of $x_6$, (—) simulated profile of $x_6$,
(ο) Experimental profile of $x_3$, (—) simulated profile of $x_3$,
C, F, I, L: (∗) Experimental profile of $X_v$, (—) simulated profile of $X_v$,
(∗ ο) Experimental profile of $x_4$, (—) simulated profile of $x_4$,
(—) simulated profile of $x_5$, unknown substrate
A model describing the progression of apoptosis was developed for Chinese Hamster Ovary cell culture. The model predicts the dynamic behaviour of caspase activation via two pathways: intrinsic and extrinsic. An unidentified substrate was correlated with nutritional stress, triggering the intrinsic pathway described by caspase-9 activation. This unknown substrate could be a growth factor in serum rather than in basal medium. Future work aims to confirm the presence of this unknown substrate in serum. Although, the combined model may require further parameter refinement it was shown that it can predict correctly metabolic data that was not used for model calibration. The model will be used to design a feeding strategy for fed-batch culture for regulating the progression of apoptosis.

REFERENCES
