EXHIBIT 4

MINI-REVIEW

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Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals

Abstract There has been a rapid increase in the number and demand for approved biopharmaceuticals produced from animal cell culture processes over the last few years. In part, this has been due to the efficacy of several humanized monoclonal antibodies that are required at large doses for therapeutic use. There have also been several identifiable advances in animal cell technology that has enabled efficient biomanufacture of these products. Gene vector systems allow high specific protein expression and some minimize the undesirable process of gene silencing that may occur in prolonged culture. Characterization of cellular metabolism and physiology has enabled the design of fed-batch and perfusion bioreactor processes that has allowed a significant improvement in product yield, some of which are now approaching 5 g/L. Many of these processes are now being designed in serum-free and animal-component-free media to ensure that products are not contaminated with the adventitious agents found in bovine serum. There are several areas that can be identified that could lead to further improvement in cell culture systems. This includes the down-regulation of apoptosis to enable prolonged cell survival under potentially adverse conditions. The characterization of the critical parameters of glycosylation should enable process control to reduce the heterogeneity of glycoforms so that production processes are consistent. Further improvement may also be made by the identification of glycoforms with enhanced biological activity to enhance clinical efficacy. The ability to produce the ever-increasing number of biopharmaceuticals by animal cell culture is dependent on sufficient bioreactor capacity in the industry. A recent shortfall in available worldwide culture capacity has encouraged commercial activity in contract manufacturing operations. However, some ana-
lysts indicate that this still may not be enough and that future manufacturing demand may exceed production capacity as the number of approved biotherapeutics increases.

Introduction

Although animal cell cultures have been important at a laboratory scale for most of the last 100 years, it was the initial need for human viral vaccines in the 1950s (particularly for poliomyelitis) that accelerated the design of large-scale bioprocesses for mammalian cells (Kretzmer 2002). These processes required the use of anchorage-dependent cells and the modern version of this viral vaccine technology currently employs microcarrier support systems that can be used in pseudosuspension cultures designed in stirred tank bioreactors.

However, more recently the enhanced interest in mammalian cell culture bioprocesses is associated with recombinant protein technology developed in the 1970s and 1980s. The first human therapeutic protein to be licensed from this technology in 1982 was recombinant insulin (Humulin from Genentech) but the relative structural simplicity of this molecule allowed its large-scale production to be developed in *Escherichia coli*, which is fast growing and robust compared to mammalian cells. It was soon realised that the subsequent targets for recombinant therapeutics were more complex and required the post-translational metabolic machinery only available in eukaryotic cells. At the present time there are up to 30 licensed biopharmaceuticals produced from mammalian cell bioprocesses (Walsh 2003; Pavlou 2003, Molowa and Mazanet 2003). These are defined as recombinant proteins, monoclonal antibodies and nucleic acid-based products. Since 1996, the chimeric and humanized monoclonal antibodies have dominated this group with such blockbuster products as Rituxan, Remicade, Synagis and Herceptin (Brekke and Sandie 2003; Pavlou 2004). A chimeric antibody (e.g. Rituxan) consists of a molecular construct in which the mouse variable region is linked to the human constant region. A further step to humanizing an antibody can be made by replacement of the

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284

murine framework region, leaving only the complementarity determining regions (CDRs) that are of murine origin. These hybrid construct molecules are far less immunogenic than their murine counterparts and have serum half-lives of up to 20 days.

In this review, key factors for the recent achievements in the production of biopharmaceuticals from animal cell culture processes are discussed.

Cell line transfection and selection

The ability to produce and select for a high-producing animal cell line is key to the initial stages of the development of a cell culture bioprocess (Wurm 2004; Andersen and Krummen 2002). Chinese hamster ovary (CHO) cells have become the standard mammalian host cells used in the production of recombinant proteins, although the mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) or human-retina-derived (PER-C6) cells are alternatives. All these cell lines have been adapted to grow in suspension culture and are well suited for scale-up in stirred tank bioreactors. The advantage of CHO and NSO cells is that there are well-characterised platform technologies that allow for transfection, amplification and selection of high-producer clones. Transfection of cells with the target gene along with an amplifiable gene such as dihydrofolate reductase (DHFR) or glutamine synthetase (GS) has offered effective platforms for expression of the required proteins. In these systems, selective pressure is applied to the cell culture with an inhibitor of the DHFR or GS enzymes that causes an increase in the number of copies of the transfected genes including the target gene.

The DHFR system is routinely used with CHO cells deficient in the DHFR activity (DHFR$^-$). The target gene is delivered to the cells along with the DHFR marker gene, usually on the same plasmid vector (Gasser et al. 1982; Lucas et al. 1996). The expression vector normally contains a strong viral promoter to drive transcription of the recombinant gene and this is delivered into the cells by one of a number of possible non-viral transfer techniques. These include calcium phosphate, electroporation, lipofection or polymer-mediated gene transfer. The transfected cells are selected in media requiring the activity of DHFR for nucleotide synthesis and cell growth. Exposure of the cells to several rounds of gradually increased concentrations of the DHFR enzyme inhibitor, methotrexate, promotes amplification of the DHFR and the co-transfected target gene. Methotrexate treatment enhances specific protein production following an increased gene copy number, which can be up to several hundred in selected cells.

The glutamine synthetase expression system is an alternative that works as a dominant selectable marker, which is an advantage because this does not require the use of specific mutant cells (Bebbington et al. 1992). CHO cells that contain endogenous enzyme activity can be used although NS0 cells are preferred because of the absence of GS. This means that a lower activity of an enzyme inhibitor can be used for selection and amplification in NS0. GS enzyme allows the synthesis of glutamine intracellularly and so the transfected cells are selected in a glutamine-free media. The added advantage of this is that the cell cultures produce less ammonia, which is a potentially toxic metabolic by-product of mammalian cells that affects protein glycosylation and may inhibit cell growth. Gene amplification in this system is mediated by methionine sulphoximine (MSX), which is required at concentrations of 10–100 μM to provide clones with amplified genes and sufficiently high specific productivities. Typically copy numbers of only four to ten genes per cell are found in these cells but they give as high expression levels as the cells from the DHFR system. The advantage of this is that the GS high-producer clones can be produced in around 3 months, which is half the time it takes for the selection of DHFR clones.

High yields of recombinant proteins can also be produced from a human cell line, notably PER.C6, which was created by immortalizing healthy human embryonic retina cells with the E1 gene of adenovirus (Jones et al. 2003). This cell line has been well characterized and has been shown to be able to produce high levels of recombinant protein with relatively low gene copy numbers and without the need for amplification protocols. The added value of these cells is that they ensure the recombinant proteins produced receive a human profile of glycosylation.

Screening for a high-producer clone can be a lengthy process that depends upon assaying the secreted proteins to determine productivities of all candidate clones. High-throughput selection systems have been devised based on rapid assays or the use of flow cytometry to identify clones that have an appropriate product marker on the cell surface (Borth et al. 2001; Carroll and Al-Rubeai 2004). A cell clone with a specific productivity of up to 10 pg cell^{-1} day^{-1} can be produced fairly routinely for recombinant protein production. However, higher specific productivity (up to 90 pg cell^{-1} day^{-1}) may be possible with improvements in vector technology and further understanding of the parameters that control protein expression in the cell (Wurm 2004).

Stability of gene expression

The stability of selected clones over long-term culture is a critical parameter for commercial production (Kim et al. 1998). The application of selective pressure such as methotrexate in the case of the DHFR selection system causes gene amplification but a proportion of these genes are unstable and removal of the selective agent, as is necessary in production cultures, results in a gradual loss of the gene copy number. Fann et al. (2000) reported the stepwise adaptation of tissue plasminogen activator-producing CHO cell lines to 5 μM of methotrexate, which resulted in a maximum specific recombinant protein production of 43 pg cell^{-1} day^{-1}, but on removal of the methotrexate the maximum productivity decreased to 12 pg cell^{-1} day^{-1} within 40 days. This decrease in productivity could be correlated

with a reduction of gene copy number for individual clones. Barnes et al. (2004) studied the stability of antibody expression from NS0 cells amplified with the GS system. They reported that there was a loss of mRNA for the recombinant protein over long-term culture but this was only reflected in a decrease in protein expression if the mRNA was below a threshold level. This indicates that selection for clones for high levels of recombinant mRNA may be useful as a predictor of stable protein production. Above a saturation level of mRNA it is argued that the limitation to protein expression resides in the translational/ secretory machinery of the cell.

Production of a high-producer clone is dependent on the integration of the expression vector in the host cell genome. The site of integration has a major effect on the transcription rate of the recombinant gene and progressive gene silencing can occur over successive culture passages after clonal isolation. This is thought to be associated with the spread of heterochromatin structure, which is condensed and transcriptionally silent. Most expression systems cause random transgene integration into the host cell and this leads to positional effects that cause variable expression and stability. However, the genetic control elements that are responsible for establishing a transcriptionally active transgene are not fully understood. Vectors in which the genes are flanked with insulators, boundary elements or ubiquitous chromatin opening elements may promote stable expression by insulating the transgene from positional effects of the chromatin. These elements that can be incorporated into expression vectors include matrix (or scaffold) attachment regions (MAR or SAR) that allow open chromatin structure to be maintained. This can allow higher efficiency of expression of the integrated genes (Kim et al. 2004). Gene regulatory elements associated with ubiquitously expressed housekeeping genes have been recently isolated (Antoniou et al. 2003). These regulatory elements appear to confer a dominant chromatin opening function and give rise to an ability to resist transgene silencing. These ubiquitous chromatin opening elements (UCOE) have also been incorporated into transgene vectors to prevent gene silencing and give consistent, stable and high-level gene expression irrespective of the chromosomal integration site (Haines et al. 2004).

Culture modes

A producer cell clone may be grown in batch cultures to above 10^6 cells/mL over 3–4 days to allow synthesis and product secretion. The limits for growth and production are related to the accumulation of metabolic by-products, such as ammonia and lactate, or the depletion of nutrients, such as glucose or glutamine (Butler and Jenkins 1989). The growth of cells can be extended if these limitations are addressed through perfusion culture in which the constant supply of nutrients and the removal of media can lead to cell densities of at least 10^7 cells/mL (Butler et al. 1983). These principles have been extended to fed-batch cultures, which have been shown to be operationally simple, reliable and flexible for multi-purpose facilities (Bibila and

Robinson 1995, Cruz et al. 2000; Xie and Wang 1997). The most successful strategies involve feeding concentrates of nutrients based upon the predicted requirements of the cells for growth and production. This can involve slow feeding of low concentrations of key nutrients. The maintenance of low concentration set points of the major carbon substrates enables a more efficient primary metabolism with leads to lower rates of production of metabolic by-products, such as ammonia and lactate. As a result the cells remain in a productive state over extended time frames. The strategic use of fed-batch cultures has enabled considerable enhancement of yields from these processes. This is often combined with a biphasic strategy of production in which cell proliferation is allowed in the first phase so that high cell densities accumulate, followed by a phase in which cell division is arrested to allow cells to attain a high specific productivity. In this type of strategy, growth can be arrested by a decrease in culture temperature (Fox et al. 2004; Yoon et al. 2003). By directly supplying cells with a balanced nutrient feed, a fed-batch culture can now be expected to yield upwards of 2 g/L of recombinant protein, which is probably at least tenfold higher than the maximum that could be expected by a simple batch culture in standard culture medium.

Animal cell cultures are normally grown in stainless steel, stirred tank bioreactors that are designed with impellers that minimize shear forces (Kretzmer 2002). Producer cells can be made to be sufficiently robust in this environment if they are provided with suitable growth media and gas sparging is carefully controlled. The capacity of commercial bioreactors for animal cells has gradually increased over the past two decades, with capacities now reported up to 20,000 L from some of the larger biopharmaceutical companies. Airlift bioreactors have also been applied to large-scale animal cells and these have been shown to be efficient for protein production.

Perfusion cultures are more demanding to set up at a large scale but they have the potential advantage of allowing a continuous stream of product over several weeks or even months (Mercille et al. 2000). A further advantage is the rapid removal of any potentially labile products from the culture environment. An effective cell separator will allow the protein-containing media to be fed directly into a chromatography column suitable for extraction and downstream processing (Shirgaonkar et al. 2004; Castilho and Medronho 2002; Wen et al. 2000). A further advantage of this mode of culture is that the bioreactor may be up to ten times smaller for the production of the same quantity of product (Ryll et al. 2000).

This area of bioprocess design will become of even greater importance as some of the first-generation blockbuster drugs (e.g. erythropoietin, human growth hormone and α-interferon) start being produced as generics (Walsh 2003). Eleven biopharmaceuticals with combined annual sales of $13.5 billion lose patent protection in 2006 (Walsh 2003). The challenge then will be to produce bioequivalents in efficient low-cost bioprocesses.

There are several challenging areas of bioprocess development that are required to be addressed to ensure the future success of animal cell culture processes. These in-

286

clude serum-free media, apoptosis, glycomics and the capacity crunch.

Serum-free media

Bovine serum was used as a supplement of cell culture media for several decades. It is a rich source of hormones, growth factors and trace elements that promote rapid cell growth and also its high albumin content ensures that the cells are well protected from potentially adverse conditions such as pH fluctuations or shear forces. However, the composition of serum is variable and undefined, which leads to inconsistent growth and productivity. Early attempts to develop serum-free substitutes incorporated such components as insulin, transferrin, albumin and cholesterol. However, the mad cow crisis in the beef industry alerted a concern for the use of animal serum and any other animal-derived components in the production of biotherapeutics. This has now led to a strong demand for cell culture formulations that are free of all animal components. The challenge that this demand poses is to be able to identify effective substitutes for all the growth-promoting factors that are present in serum. It turns out that producer cell lines are quite fastidious in their growth requirements and that such requirements vary considerably from one cell line to another. Therefore, it has not been possible to design a single serum-free formulation to act as a serum substitute suitable for the growth of all cell lines. In fact even different clones of CHO cells may require different formulations for optimal growth. This has given rise to a strong drive for the development of serum-free and animal-component-free formulations that are tailored to the needs of specific producer cell lines.

There are several strategies that can be used to design these formulations. Combinations of standard basal media may be tested to determine those that result in good cell growth and productivity at minimal serum levels. In some cases metabolic analysis may help in media design. For example, NS0 myeloma cells lack a functional pathway for cholesterol synthesis and so cholesterol is required as a lipoprotein supplement in the medium (Gorfien et al. 2000). Protein hydrolysates from non-animal sources have been found to provide good growth promotion in some culture systems (Sung et al. 2004). Analysis of the depletion of media components may lead to the identification of specific nutrients that may be required at higher supplement levels or for inclusion in feeding regimes. Statistical approaches can be used, such as the Plackett–Burman experimental design, so that mixtures of components may be tested simultaneously in matrix experiments of growth and productivity (Castro et al. 1992). Another original approach is the identification by microarray analysis of specific receptors expressed during cell growth, so that corresponding ligands may be incorporated into the medium (Donahue 2004). These approaches are presently being used by various specialized media companies to customise animal-component-free formulations for the production of the plethora of recombinant proteins that are being introduced into the market.

Apoptosis

Cell cultures are often terminated because of cell death that may be caused by one of several factors including nutrient depletion, metabolic by-product accumulation, excessive shear forces or hypoxia. Cell death may be by necrosis caused by extreme conditions resulting in physical damage to the cells. Alternatively and more commonly, cell death in a bioreactor occurs by apoptosis, which is a form of programmed cell death regulated through a cellular cascade of activities in response to one of the factors mentioned above (Arden and Betenbaugh 2004). Characteristic changes include chromatin shrinkage followed by membrane blebbing and the formation of apoptotic bodies. The DNA of the cell is fragmented and this can be the basis of an assay to quantify apoptosis in a cell population.

It is of considerable value to be able to prevent or inhibit apoptosis in culture in order to extend the time of high cell viability and prolong protein production. There are two strategies that can be used for this. The cellular environment can be manipulated through media supplementation or the intracellular environment can be modified by genetic engineering.

Nutrient feeding can provide protection and this is normally used as the first preventive measure to control the cellular environment to delay apoptosis. Serum is known to contain unidentified anti-apoptotic factors that can offer protection (Zanghi et al. 1999). However, the serum-free formulations that are required for production processes make the cells more vulnerable to apoptosis. Some supplements such as suramin (Zanghi et al. 2000) or insulin growth factor (Sunstrom et al. 2000) may provide independent anti-apoptotic protection in serum-free cultures. There are also other specific caspase inhibitors available to suppress apoptosis (Tinto et al. 2002) but their expense in large-scale cultures is likely to be prohibitive.

Genetic strategies involve the transfection of anti-apoptotic genes such as bcl-2 or bcl-x_2 into a host cell. The expression of the corresponding proteins inhibits the release of pro-apoptotic molecules from the mitochondria and may prolong the viability of the cell. This strategy has been shown to work for several cell lines, which have shown higher viabilities and improved robustness under conditions that would normally be expected to cause apoptosis (Tey et al. 2000; Mastrangelo et al. 2000; Kim and Lee 2002).

Glycosylation

Animal cells are used for biomanufacture because of their capabilities of adding carbohydrates (glycans) to synthesised proteins (Butler 2004). These are produced as pools of different glycoforms with varying glycan structures attached to a single peptide backbone with a known amino acid sequence. The basic protein structures can be controlled and directed by the expression of appropriate genetic sequences. However, controlling the pool of glycan structures (glycomics) that occupy a recombinant protein is still difficult. Variations may be found in the site occupancy (mac-

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