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ORIGINAL PAPER

# Regulating apoptosis in mammalian cell cultures

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**Abstract** Cell culture technology has become a widely accepted method used to derive therapeutic and diagnostic protein products. Mammalian cells adapted to grow in bioreactors now play an integral role in the development of these biologicals. A major limiting factor determining the output efficiency of mammalian cell cultures however, is apoptosis or programmed cell death. Methods to delay apoptosis and increase the longevity of cell cultures can lead to more economical processes. Researchers have shown that both genetic and chemical strategies to block apoptotic signals can increase cell culture productivity. Here, we discuss various strategies which have been implemented to improve cellular viabilities and productivities in batch cultures.

**Keywords** Mammalian cell culture · Apoptosis · Bcl-2 protein · Cell cycle arrest · Recombinant protein production

## Introduction to mammalian cell culture technology

Today, the biotechnology and pharmaceutical industrial sectors rely heavily on mammalian cell cultures as bio-production systems to manufacture various biological therapeutics including antibodies, interferons, hormones, erthyropoetin, clotting factors, immunoadhesins, and vaccines. The market for monoclonal antibodies alone is expected to grow 30% a year and reach sales of over 6.5 billion by 2006. The vast majority of these biological therapeutics are secreted glycoproteins obtained from mammalian cell lines such as Chinese Hamster Ovary (CHO), Human Embryonic Kidney (HEK-293) and NS/0 (murine myeloma) cells. As is the goal with most commercial products, economical methods are desired to deliver large amounts of product. Biotechnologists, therefore, strive to generate these valuable secreted glycoproteins in the highest yields possible in order to utilize mammalian bioreactor facilities efficiently.

The use of large-scale bioreactor vessels is a standard industrial feature for the storage of suspension cell cultures. Large-scale industrial bioreactors can hold cell culture volumes up to 20,000 l. The scale-up processes for these systems generally begin in stirred-tank batch reactors. Selection of bioreactor vessels and methodologies for mammalian cell growth and maintenance are essential in the cell culture and scale-up processes. Presently, fed-batch and perfusion reactors are favored over batch cultures due to the accumulation of metabolic byproducts and nutrient depletion which occurs more rapidly in batch

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systems limiting both growth and production conditions. Bioreactor performance can be improved both by increasing viable cell densities and by enhancing the productivities of individual cells within the reactors. Unfortunately, both of these strategies will subject the cells to considerable external and internal stresses. Cells grown to high densities in bioreactors can be exposed to nutrient limitations or toxic byproducts, even in fed-batch and perfusion bioreactors. Furthermore, the secretion of very high levels of recombinant protein will tax the individual cell's capacity to fold, glycosylate, process, and secrete complex glycoproteins.

Both the internal stress of processing very high levels of non-native polypeptides and the external strain of nutrient limitations and environmental toxins can activate the mammalian cell stress response machinery leading to inhibition of protein synthesis, suppression of cell growth, and programmed cell death, also called apoptosis (Arden and Betenbaugh 2004). Apoptosis is a regulated physiological process in which cells respond to particular external and internal stimuli by activating a cascade of biochemical pathways that end in the cell's own demise. While suppression of growth or apoptosis may be appropriate responses in vivo to prevent the onset of cancer, these responses can have harmful consequences for mammalian cells grown in culture (Kerr et al. 1972; Raff 1998). Cell growth arrest can reduce final cell densities achieved in bioreactors and apoptosis will eliminate valuable viable producer cells and terminate a bioreactor run. The suppression of protein synthesis is also undesirable, as this response will lower the productive capacity of the individual producer cells. The cumulative effect of a cell's response to internal and external stresses is a decline in bioreactor productivity and a reduction in final protein yields.

## Cellular engineering

Extracellular and intracellular engineering strategies can be implemented to limit the undesirable activation of the cellular stress response. The fortification of culture medium and adoption of fed-batch or perfusion systems can increase nutrients and lower accumulations of toxins. However, the adoption of these methodologies is not sufficient to eliminate completely stress-induced signaling. Therefore, a complementary cell engineering strategy can be applied to control the intracellular response to external and internal stress signals. With this method, the cell's internal physiology is altered in order to limit or prevent the activation of undesirable response pathways to stress. Such a cell engineering approach can be achieved through an understanding of the various stress-induced pathways involved, which are discussed below. Where genetically engineering a cell by changing its gene expression leads to altered cell physiology. This method of genetic engineering can be used to study the effects of a certain gene or genes on a cell or to change or improve cell performance. Combined molecular biology and cell culture techniques have led to the numerous advancements in this area of cell engineering.

Different gene expression systems are available to study cellular effects of gene overexpression. Often, researchers will introduce a constitutively expressing gene into the host cell to observe changes in cellular genome expression, morphology, and physiology. Introducing a desired gene with an active promoter into the host cell chromosome ensures that the gene will be transcribed continuously. However, a limitation to this approach is that frequently, genes may be expressed more in some clones than in others (Tey and Al-Rubeai 2004). This limitation can usually be overcome by the examination of multiple clones and control cells. In other cases, controlled gene expression is preferred. Such controlled expression systems function as a ''switch'' for the gene of interest and such technologies are called inducible gene expression systems. In cases where inducible systems are preferred, the desired gene being expressed may be harmful to the cell if constitutively expressed. Another reason for using inducible systems is having more control over when the gene is expressed so that researchers can observe how the cell responds when the specific gene is turned on or off.

## Apoptosis

Two general modes of cell death have been characterized and are known as necrosis and apoptosis. Necrosis results from extreme conditions that cause sudden acute damage to cells leading to swelling and rupture with release of cellular contents into the surroundings. Apoptosis, however, is a regulated physiological response resulting from an initially non-lethal stimulus that activates a molecular cascade of events culminating in the cell's demise (Table 1). To optimize cell viabilities and protein yields in culture much attention is being given to strategies for controlling cell death.

Apoptosis is a genetically controlled process and can be morphologically recognized by cell and chromatin shrinkage followed by plasma membrane blebbing (Fig. 1). Blebbing involves the shedding of membrane fragments from the whole in the form of apoptotic bodies that often include cytosolic and nuclear contents. Apoptotic Chinese hamster ovary (CHO) cells exhibiting membrane blebbing are compared to wild-type CHO cells expressing the GFP protein (Fig. 2A, B). These apoptotic bodies can be phagocytosed in vivo although in vitro they may break apart or accumulate during the cell culture process. An understanding of the molecular mechanisms of apoptosis and the factors that control it can be applied to inhibit cell death and improve bioreactor performance.

## Apoptotic pathways

Cells respond to both extracellular and intracellular stress through the initiation of signaling cascades that eventually lead to either cell cycle arrest, inhibition of protein synthesis, or, in severe cases, apoptosis. There are three apoptosis pathways that respond to the different apoptotic stimuli that result in a similar fate such as: (1) death receptor or receptor-mediated, (2) mitochondrial and (3) endoplasmic reticulum (ER) (Fig. 3). These individual pathways are initiated by specific signals that activate a biochemical cascade ultimately resulting in apoptosis. Furthermore, these pathways include cross-communication that can lead to the activation of multiple pathways simultaneously (Adams 2003; Arden and Betenbaugh 2004).

## Caspase proteases

Apoptosis involves the activation of cysteine–asparate proteases, or caspases. Caspases are the key proteases responsible for the morphological changes occurring during cellular degradation and are activated for all stress pathways that initiate apoptosis. Inactive caspases, referred to as procaspases, are present in normal cells in their inactive zymogen state. The activation of these pro-caspases occurs through the cleavage of the pro domain either as a result of auto-activation or from cleavage by another cellular caspase. Each stress pathway has specific initiator caspases that are particular to that specific apoptosis cascade: caspase-8 from the death receptor mediated pathway, caspase-9 from the mitochondrial pathway, and possibly caspase-12 from the ER pathway. These initiator caspases are activated first in a particular stress pathway and subsequently activate downstream effector caspases. The

**Table 1** A comparison of characteristic behavior in necrosis and apoptosis, the two modes of cell death

| Necrosis | Apoptosis |
|---|---|
| Abnormal tissue death | Programmed cell death |
| Spontaneous, does not require energy | Controlled, requires ATP |
| Cell swells, inflammation | Cell shrinkage, no inflammation |
| DNA fragments into random sizes | DNA fragments into specific sized multiples of 185 bp |
| Loss of plasma membrane integrity due to lysis | Plasma membrane undergoes blebbing but remains intact |
| Cell contents release to surrounding environment | Budding off of apoptotic bodies (cell contents encapsulated) |
| In multicellular organisms, cell debris is cleared by macrophages | In multicellular organisms, apoptotitc bodies are phagocytosed |

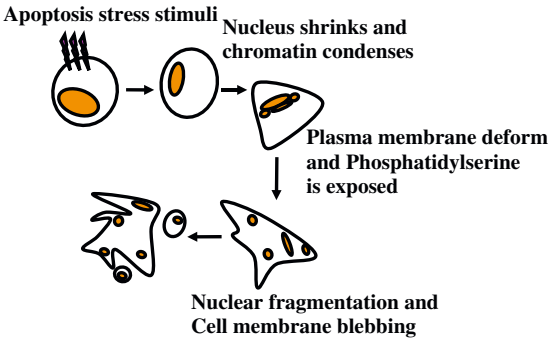


**Fig. 1** Schematic describing the various morphological features associated with apoptosis. An initially healthy cell encounters either intracellular or extracellular stress leading to the initiation of apoptosis which is followed by cell and nuclear shrinkage. As the cell progresses to later stages of apoptosis, the plasma membrane begins to deform and the membrane lipid, phosphatidylserine is exposed. This is followed by nuclear fragmentation and the breaking off of the cell membrane also referred to as blebbing

effector caspases, including caspase-3, caspase-6, and caspase-7, are the proteases, which are both directly and indirectly responsible for cellular degradation. The biochemical and morphological changes associated with apoptosis include chromosomal condensation, membrane blebbing, and DNA fragmentation (Ashkenazi 2002; Cohen 1993; Cummings et al. 1997; Sauerwald and Betenbaugh 2002). Some of these morphological changes are caused directly by caspase degradation while others are the result of enzymes activated by caspases. For example, DNA fragmentation is catalyzed by a DNAse that is activated following the caspase cleavage of a separate DNAse inhibitor.

## Death receptor pathway

The death receptor-mediated apoptotic pathway is triggered by the binding of ligands to specific receptors of the tumor necrosis factor (TNF) superfamily at the cell plasma membrane (Ashkenazi 2002; Strasser et al. 2000). Ligand binding to these receptors promotes the recruitment of the protein, Fas-associated death domain (FADD) onto the cytoplasmic tail of the receptors followed by the recruitment of initiator procaspase-8, and caspase-10 in humans (Kischkel et al. 2001; LeBlanc and Ashkenazi 2003) (Fig. 3). The proximity of zymogens promotes dimerization and autocatalytic activation of the initiator caspases, which then activate the effector caspases including caspase-3, −6, and −7 (Boatright and Salvesen 2003). The death-receptor pathway also communicates with the mitochondrial pathway through the small pro-apoptotic Bcl-2 homolog, Bid, which is activated by caspase-8 cleavage to generate truncated Bid (tBid) (Zha et al. 2000). Activated tBID translocates to the mitochondrial membrane, where it creates pores and stimulates the activation of the mitochondrial apoptosis cascade through an interaction with the pro-apoptotic Bax or Bak proteins (Scorrano et al. 2003).

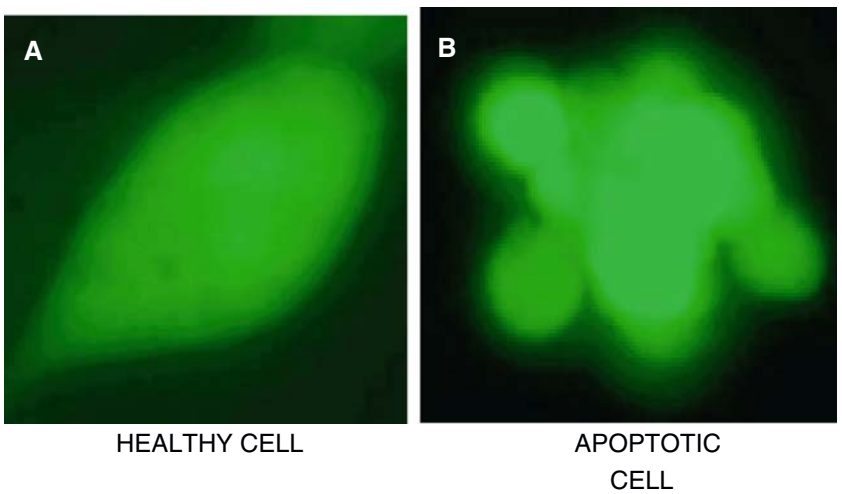


**Fig. 2** CHO cells transfected with GFP show the morpholigical features associated with (**A**) a healthy mammalian cell and (**B**) an apoptotic cell in which the plasma membrane has deformed and is breaking off into small apoptotic bodies or blebbing

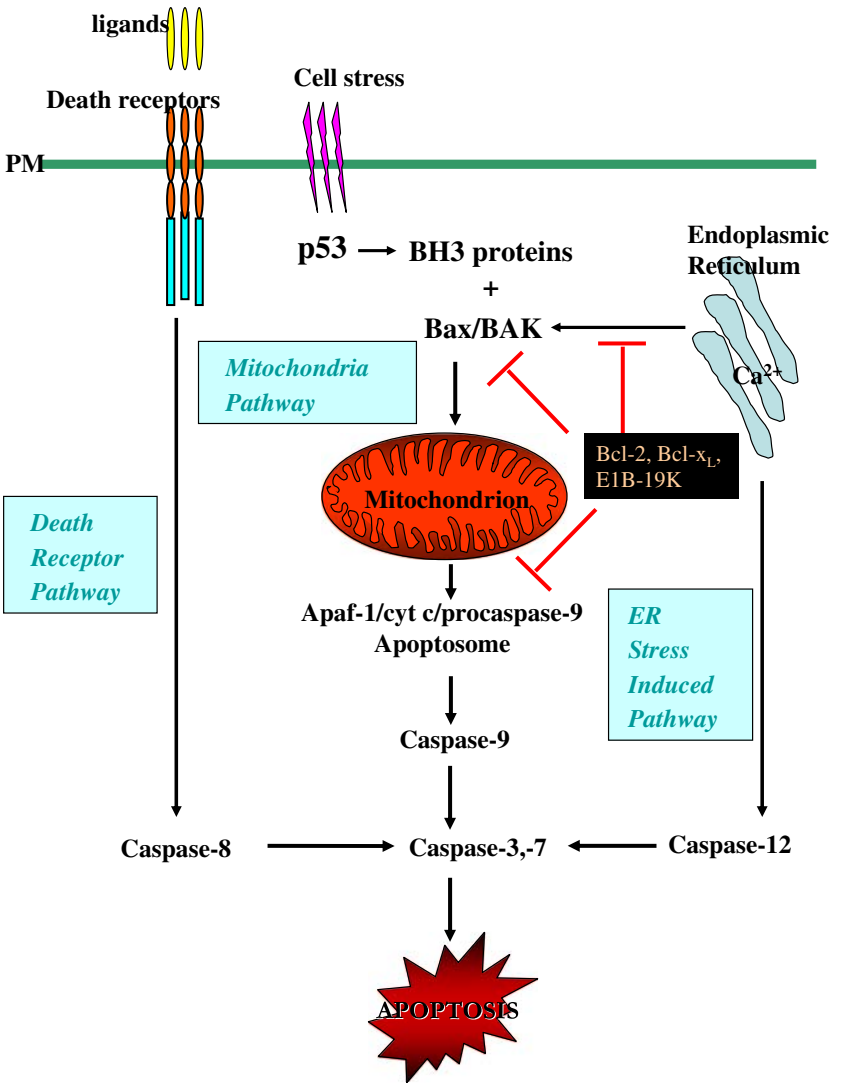


**Fig. 3** Schematic of the apoptotic signaling pathways. The death receptor pathway is intiated by external stress signals and initiates caspase activity. The endoplasmic reticulum can undergo an unfolded protein response which leads to stress signals from the ER to the cytoplasmic space and initiates caspase activity. The mitochondrial stress pathway is initiated by various intracellular and extracellular stresses that lead to permeation of the mitochondrial membrane followed by the release of pro-apoptotic proteins localized within the mitochondria into the cytoplasmic space

**Mitochondrial stress pathway**

Mitochondrial dysfunction caused by permeation of the mitochondrial membrane is a key indicator that the apoptotic program is underway. The mitochondria house a variety of pro-apoptotic proteins. The release of these proteins into the cytosolic space leads to the activation of multiple pro-apoptotic factors. Apoptosis occurring due to mitochondrial dysfunction is often referred to as the intrinsic pathway since its activation is initiated by signals from the intracellular environment. This does not mean, however, that the initial stress stimuli must come from the intracellular space. Nutrient or growth factor deprivation, UV radiation, exposure to toxins such as etoposide, hypoxia, or genetic damage can all initiate the intrinsic mitochondrial pathway, sometimes through p53 amplification, or through the activation of the initiator caspase-9 (Cory et al. 2003). The critical initiating event in caspase-9 activation and apoptosis is the release of cytochrome *c* and other pro-apoptotic molecules such as Smac/Diablo, Om/HtrA2, endonuclease G, and AIF from the mitochondria (Adams 2003; Arden and Betenbaugh 2004). Cytochrome *c* binds with cytoplasmic Apaf-1 and procaspase-9 to form a complex known as the apoptosome which activates caspase-9, leading to activation of downstream effector caspases that are

responsible for cleavage of cytoplasmic and nuclear substrates.

The Bcl-2 family of proteins, grouped into three distinct subfamilies, play critical roles in both promoting and opposing the release of cytochrome *c* (Cory et al. 2003; Scorrano et al. 2003). Bcl-2 protein and its closest homologs including Bcl-$x_L$ and Bcl-w (Bcl-2 subfamily) include four conserved Bcl-2 homology (BH1 to BH4) domains. A second group of Bcl-2 family members, including Bax and Bak (Bax subfamily), are strongly pro-apoptotic and include homology to the Bcl-2 family in the BH1 to BH3 domains (Scorrano et al. 2003). The third BH3 subfamily consists of Bik, Bid, Bad, and numerous others that retain homology to Bcl-2 only within the BH3 region (Cory et al. 2003; Scorrano et al. 2003).

Members of this large BH3 subfamily are also pro-apoptotic and represent the key upstream sensors of apoptotic signals within the cells (Cory et al. 2003; Scorrano et al. 2003). They promote apoptosis possibly through binding to their anti-apoptotic relatives including Bcl-2, Bcl-$x_L$, and Bcl-w or, in some cases, to pro-apoptotic relatives Bax or Bak, two critical positive regulators of mitochondrial apoptosis in mammalian cells (Cory et al. 2003; Kelekar et al. 1997; Oda et al. 2000; Scorrano et al. 2003; Yang et al. 1995). Binding of BH3 proteins results in activation of Bax or Bak leading to formation of membrane-associated homo-oligomers that facilitate release of pro-apoptotic mediators (Cheng et al. 2001; Cory et al. 2003; Lindsten et al. 2000; Scorrano et al. 2003; Zong et al. 2001). The Bcl-2 subfamily opposes apoptosis by a mechanism that is not yet fully understood. One way in which Bcl-2 may prevent apoptosis is by ''titrating'' BH3 molecules and thereby preventing Bax and Bak oligomerization (Adams 2003). Bcl-2 may also play a critical role in altering membrane permeability to pro-apoptotic factors. Once released, cytochrome *c* binds to Apaf-1 forming heptamers with pro-caspase-9, followed by auto-activation of the zymogen (Adams and Cory 2001; Liu et al. 1996). Caspase-9 then activates the effector caspases-3, −6, and −7 responsible for cell degradation and death.

## ER stress pathway

The endoplasmic reticulum (ER) is a key intracellular sensor detecting stress signals that may culminate in cell cycle arrest or apoptosis (Adams 2003; Arden and Betenbaugh 2004; Breckenridge et al. 2003; Cudna and Dickson 2003; Ferri and Kroemer 2001; Kaufman 2002; Rao et al. 2004). The ER is responsible for processing secreted and membrane proteins and is often subject to severe stresses resulting from improper glycosylation, misfolded proteins, protein aggregation, glucose deprivation, or fluctuations in calcium homeostasis (Adams 2003; Breckenridge et al. 2003; Cudna and Dickson 2003; Kaufman 2002; Kaufman et al. 2002). Stress-inducing events are further amplified in mammalian cell lines that are selected for high-level production of secreted biotherapeutics. Thus the ER represents one starting point for intracellular stresses in these cells. This is particularly true since ER stress activates what is called the unfolded protein response (UPR) leading to a series of intracellular signaling cascades (Cudna and Dickson 2003; Kaufman 2002; Patil and Walter 2001; Ron 2002). ER stress can cause cell cycle arrest by inhibiting protein translation or apoptosis through both mitochondrial-independent or -dependent mechanisms. Proteins localized to the ER act as stress sensors and can activate caspase-12 or other caspases in mammalian cells leading to mitochondrial-independent apoptosis by activating downstream caspases and ultimately cell degradation. The mitochondrial-dependent pathway results from fluctuations in cytoplasmic $Ca^{2+}$ levels that lead to the activation of pro-apoptotic BH3 family members and their subsequent translocation to the mitochondrial membrane that leads to apoptosis. $Ca^{2+}$ levels in the ER and cytosol may also be controlled by the presence of Bcl-2 and Bax subfamily apoptotic members that accumulate on the ER membrane (White et al. 2005; Zong et al. 2003).

## p53 Tumor suppressor

As the energy powerhouse of the cell, mitochondria represent a critical organelle, involved

in nearly all functions of the cell. The degradation or deactivation of mitochondria will ultimately lead to necrosis or apoptosis. Upstream stresses can lead to mitochondrial membrane permeation and degradation. These stresses sometimes involve the activation of a tumor suppressor protein known as p53. Cellular p53 levels increase in response to various stresses including; UV light, hypoxia, DNA damage, and heat shock resulting in either cell cycle arrest or apoptosis (Weinberg et al. 2004). Upon upregulation, p53 becomes stabilized and acts as a transcription factor for many genes, binding to consensus sequences found on DNA with its central core domain of 200 amino acids (Cho et al. 1994). The mechanism by which p53 functions is thought to involve tetramerization of the protein at the C-terminus which containing a tetramerization domain (Fig. 4) (Nicholls et al. 2002). Under normal and healthy cellular conditions, p53 and its homologs, p63 and p73, are unstable, undergoing rapid degradation to maintain a short half-life. However, various types of cellular stress lead to phosphorylation of p53 and its homologs by ATM, Chk1 or Chk2, stabilizing these proteins and thereby allowing them to act as transcription factors for cell cycle arrest genes or apoptotic genes (Bhonde et al. 2006; Gueven et al. 2006). Depending on the stress type and severity, p53 stabilization may cause cell cycle arrest through upregulation of kinase inhibitor $p21^{Cip}$ (Huo et al. 2004; Sak et al. 2003; Zhang et al. 2005). However, when stress stimuli are continual and the cell is unable to repair damage through cell cycle arrest, p53 activation induces apoptosis through mitochondrial stress and degradation following the upregulation of BH3 anti-apoptotic proteins including; Bax, Noxo, Puma and others (Chipuk and Green 2006; Oda et al. 2000; Sanfeliu and Stephanopoulos 1999; Scorrano et al. 2003; Zhang 2006). Therefore the decision between growth arrest and apoptosis relies on the dominant gene targets of p53. Stress induced p53 activation is a leading cause of apoptosis and has been largely overlooked by biotechnologists. Genetically modifying cells to silence or limit p53 function effectively inhibits apoptosis pathways before they begin may be an appropriate future target.

## Methods to detect apoptosis

Assays to detect apoptosis in cell populations include measuring DNA fragmentation through DNA ladders, a signature of apoptosis, or detecting activation of apoptosis-induced proteases such as caspase-3, an initiator caspase, or poly(ADP-ribose)polymerase (PARP) which is involved in DNA repair during apoptosis using

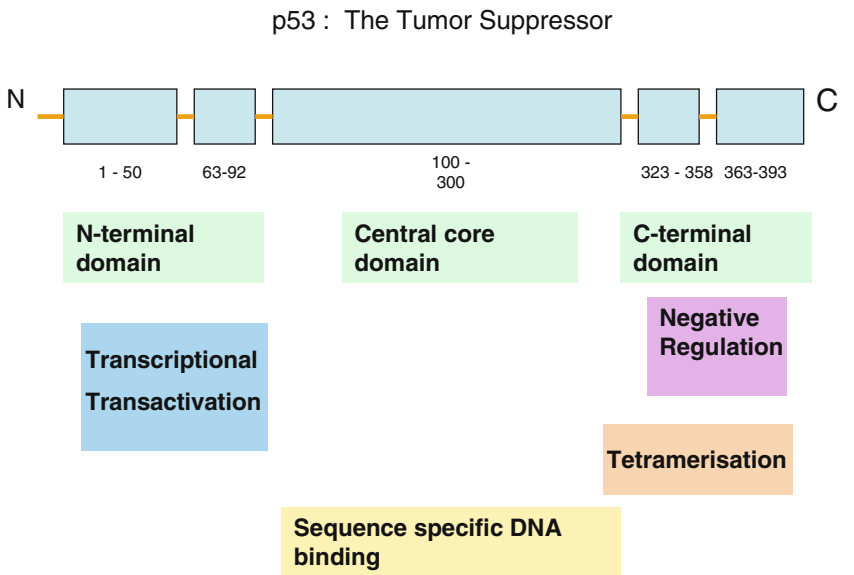


**Fig. 4** p53 protein structure consists of several domains including the N-terminal domain which is responsible for transcriptional transactivation, the central core domain which is a sequence specific DNA binding domain, and the C-terminal domain which houses a tetramerization and negative regulation domain

Western blot techniques. Methods to detect apoptosis in individual cells include terminal deoxynucleotidyl transferase nick-end-labeling, (TUNEL) labeling, Annexin V binding to cell membranes, and DNA staining with propidium iodide, DAPI, or ethidium bromide.

Since methods for early detection and quantification of apoptosis in cells are preferred, flow cytometry, a laser-based technology, is now a valuable tool in detecting programmed cell death. Cell death triggers conformational changes in the plasma membrane and translocation of phosphatidylserine to the cell surface. Annexin V is a calcium and phospholipid binding protein that can be tagged with a fluorescent marker and used to bind phosphatidylserine, which is exposed at the cell surface as one of the initial morphological changes during the apoptosis cascade. Other flow cytometric methods used to detect apoptosis include light scattering based on cell size and shape, propidium iodide exclusion of cells, analysis of organelle function such as mitochondria with rhodamine and lysosomes with acridine orange, end labeling DNA strand breaks using TUNEL or TdT and biotinylated dUTP, or cleavage of fluorogenic caspase pseudo-substrates. Detection of fluorescence and measurement of the intensity using the flow cytometer enables a quantitative evaluation of cell death. Unfortunately, the distinction between necrotic and apoptotic cell populations can be difficult for some stains. As a result one or more assays can be used to verify apoptotic cell populations.

## Strategies to inhibit apoptosis

While apoptosis is a critical event occurring during the development of multicellular organisms in vivo (Adams and Cory 1998; Raff 1998), it is now recognized as widespread in mammalian cell cultures as well. Mammalian cells, in particular, are extremely sensitive to their environment and respond to a number of internal stresses such as DNA damage or un-folded proteins and external conditions including nutrient deprivation, growth factor withdrawal, metabolic byproduct accumulation, oxygen limitations, and viral infections by activating the apoptosis cascade (Al-Rubeai et al. 1990; Chung et al. 1998; deZengotita et al. 2002; Franek and Sramkova 1996; Goswami et al. 1999; Mastrangelo et al. 1996; Mercille and Massie 1994a; Mercille and Massie 1994b; Singh et al. 1994; Singh et al. 1997). Since high levels of apoptosis are detected in mammalian cell bioreactors, blocking or delaying apoptosis represents an important biotechnological problem (Al-Rubeai and Singh 1998; Franek and Dolnikova 1991; Goswami et al. 1999; Mastrangelo and Betenbaugh 1998; Singh and Al-Rubeai 1998).

Since apoptosis leads to the loss of viable cells and thus lowers viabilities in bioreactors, methods to limit the activation and delay the onset of apoptosis cascades are being evaluated. Inhibiting or slowing the onset of cell death can increase cell longevity and lead to more productive cell culture systems (Mastrangelo and Betenbaugh 1998). Two strategies for enhancing cell survival in bioreactors include the altering of the external environment through media supplementation and manipulating the internal environment by implementing genetic modifications.

## Genetic methods to limit apoptosis

Intracellular physiology of the cell can be manipulated using genetic or cell engineering approaches in order to inhibit apoptosis. Multicellular organisms contain various proteins that act as positive and negative regulators of the apoptosis cascade. These regulatory proteins can be overexpressed to regulate apoptosis of mammalian cells in culture. This approach has shown in some instances to improve the production of recombinant proteins including antibodies for different mammalian cell lines of commercial interest.

## Bcl-2 family members

The anti-apoptotic gene, Bcl-2, is a well established inhibitor of the apoptosis cascade. This gene has been expressed in a wide range of mammalian cell lines exposed to various stresses including nutrient limitation, serum deprivation, accumulation of spent medium, exposure to toxins, irradiation, and viral infections (Charbonneau

et al. 2003; Chung et al. 1998; Cory et al. 2003; Figueroa et al. 2003; Jung et al. 2002; Kim and Lee 2002b; Mastrangelo et al. 2000a; Mastrangelo et al. 2000b; Meents et al. 2002; Perani 1998; Simpson et al. 1999; Vives et al. 2003). The expression of anti-apoptotic members of the Bcl-2 family has been shown to effectively increase cellular viabilities and in some cases enhance protein production levels as described below. Various anti-apoptotic Bcl-2 family members including Bcl-$x_L$, Bcl-w, and viral homologues including E1B-19K from Adenovirus, KsBcl-2 from Karposi's sarcoma-associated herpesvirus and BHFR-1 from Epstein–Barr virus have been expressed and shown to inhibit programmed cell death in mammalian cell culture (Jung et al. 2002). The expression of the Adenoviral Bcl-2 homologue, E1B-19K, in NS0 mouse myeloma cells increased viabilities and chimeric antibody production in perfusion culture (Mercille et al. 1999). A 3-fold decline in death rates was observed in NS0 myeloma cells expressing E1B-19K when compared to control NS0 cells in perfusion cultures. In these same NS0 cells expressing E1B-19K, monoclonal antibody (MAb) concentration increased by a factor of more than 7-fold in perfusion versus batch culture as opposed to a 3-fold increase in control NS0 cells in perfusion versus batch. The reason for the enhanced production of the engineered NS0 cultures in perfusion was due to the ability of the E1B-19K protein to enhance cell survival in long-term perfusion cultures.

Another study was conducted in CHO cells engineered to express both Bcl-2 and a chimeric antibody. An increase in viable cell number of 75% was observed in batch cultures expressing heterologous bcl-2 as opposed to control cultures. Despite increased viabilities in bcl-2 expressing cell cultures, antibody concentrations in both cell cultures gave similar production levels with titers reaching maximum levels of 40 μg $ml^{-1}$ (Tey et al. 2000b). Differing results obtained in these two studies suggest that the effect of expressing anti-apoptosis genes may depend significantly on the particular cell culture environments. Expression of anti-apoptosis genes may be most effective when applied in cultures of extended duration that occur in fed-batch or perfusion environments.

Several groups have observed that the anti-apoptotic Bcl-2 family member, Bcl-$x_L$ can perform even better than Bcl-2 in its capacity to delay the onset of apoptosis for particular insults (Chiang and Sisk 2005a, b; Mastrangelo et al. 2000b; Meents et al. 2002). The effects of Bcl-$x_L$ and Bcl-2 over-expression on protein production were evaluated in CHO cells producing sICAM, a common cold therapeutic. Expression of Bcl-$x_L$ in these cells led to enhancements in ICAM production levels from 0.21 to 0.38 mg $ml^{-1}$ in batch culture. However, the same cell line expressing Bcl-2 was shown to have much lower protein production levels of 0.02 mg $ml^{-1}$ (Meents et al. 2002). Interestingly, cellular viabilities of Bcl-$x_L$ and Bcl-2 expressing cells were closely related to the specific method of gene expression. Non-amplified expression of these genes showed minimal effects on viability, whereas methotrexate-amplified expression suppressed apoptosis in both Bcl-2 and Bcl-$x_L$ expressing cell clones. However, Bcl-$x_L$ clones showed 2-fold lower apoptotic cell levels when compared to Bcl-2 clones in cell lines with amplified expression. Such studies indicate that the particular Bcl-2 family member used, cell type, expression system as well as the culture environment can be an important factor in determining the effects of these anti-apoptosis proteins on protein production in cell culture (Chiang and Sisk 2005a).

Another alternative is the use of variants of the wild-type Bcl-2 family members. In a study conducted by Figueroa et al. 2001, CHO cells were engineered to express a Bcl-2 variant which was less susceptible to degradation. A variant of the Bcl-2 was designed by deleting the non-conserved loop region which contains potential caspase cleavage sites and forms a bridge between the BH3 and BH4 domains of the protein. To determine the effects of using this variant, CHO cells were grown in batch cultures and exposed to various insults such as glucose deprivation, spent medium and virus infection. Results indicate that CHO cells expressing the Bcl-2 variant were more viable than cells expressing the wild-type protein for all insults (Figueroa et al. 2001). This study led to creating a similar mutation in Bcl-$x_L$ gene to determine if this too would lead to higher viabilities among cells exposed to such harsh

environmental conditions. Engineered CHO cells expressing the Bcl-$x_L$ variant again were found to have improved viabilities for all conditions (Figueroa et al. 2003).

## Caspase inhibition

The activation of caspases will occur for all apoptosis cascades regardless of the particular stress-induced pathway. Despite being downstream in the apoptotic pathways, researchers have successfully inhibited caspase activity using genetic and chemical strategies in order to delay late stages of apoptosis (Mastrangelo and Betenbaugh 1998; Sauerwald et al. 2002; Sauerwald et al. 2003). The X-linked inhibitor of apoptosis (XIAP) is an inhibitor of caspases that acts downstream from Bcl-2 and Bcl-$x_L$ in the apoptosis pathway. XIAP includes three baculovirus IAP repeats (BIRs) that inhibit caspases-9, −3, and −7 and a C-terminal RING finger acting as a ubiquitin ligase. Viral protease inhibitors such as baculovirus p35 and the poxvirus cytokine response modifier CrmA, are also available to inhibit intracellular caspases (Cheng et al. 1996; Goswami et al. 1999; Komiyama et al. 1994). Consequently, cells have been engineered with the genes coding for these inhibitors in order to alter mammalian cell survival in cultures.

In order to compare the effectiveness of different caspase inhibitors, the genes for the XIAP variant containing the BIR domains (XIAP-BIR123) and the viral caspase inhibitor variant, CrmA-DQMD, were expressed in cell lines and exposed to different stresses. While BIR123 is known to inhibit caspase-9, the initiator caspase of the mitochondrial apoptosis pathway, CrmA-DQMD is an inhibitor against caspase-8, the initiator caspase of the death receptor pathway. Both proteins inhibit the executioner caspase-3. HEK-293 and CHO-K1 cells engineered to express either XIAP-BIR123 or CrmA-DQMD were exposed to a number of stresses including spent medium and etoposide, a toxin that leads to release of cytochrome *c* from the mitochondria. The caspase-9 inhibitor, XIAP-BIR123, was found to be more effective in protecting cells against etoposide exposure while CrmA-DQMD was a more effective inhibitor of cell death in spent medium. Thus the two different cell culture insults appear to trigger different apoptosis pathways for mammalian cells in culture. Furthermore the two caspase inhibitors, XIAP-BIR123 and CrmA-DQMD appeared to offer selective protection against specific cell culture insults to indicate that caspase inhibitors may be targeted at the particular stress pathways (Sauerwald et al. 2003).

## Other genetic methods

Variants of wild-type pro-apoptotic family members can also be used as vehicles for limiting the apoptotic response. A dominant-negative form of caspase-9, which binds substrates but does not facilitate cleavage, has been used to inhibit the activation of downstream caspases and the apoptotic response for several stimuli including UV irradiation (Sitailo et al. 2002). A similar approach can be applied for other initiator caspases as well as pro-apoptotic proteins such as FADD (Jendrossek et al. 2003).

## Viral proteins

Several viral proteins from adenovirus, human immunodeficiency virus-1, Karposi's sarcoma-associated herpes virus, human T-cell leukemia virus-1, hepatitis B virus, and Epstein–Barr virus stabilize the mitochondrial membrane and target; p53, certain bcl-2 family members, the permeability transition pore complex, cyclophilin D, VDAC, and the peripheral benzodiazepine receptor. Viral proteins can therefore regulate apoptosis at the mitochondrial transition level (Boya et al. 2003). In order to produce more durable hybridoma cell lines, viral bcl-2 homologues ksBcl-2 from Karposi's sarcoma-associated herpesvirus and BHFR-1 genes from Epstein–Barr virus were used in combination with apoptotic stimuli to examine the effectiveness of these proteins in delaying cell death. Cells expressing BHFR-1 provided the most protection against apoptosis induced in glutamine-free culture conditions (Jung et al. 2002). Alternatively, the E1B-19K adenoviral gene has been shown to offer

protection against apoptosis for NS0 cells exposed to nutrient limitation (Mercille et al. 1999).

### Heat shock proteins

Heat shock proteins of the Hsp 70 family function as molecular chaperones involved in protein folding, transport, and degradation. In addition, Hsp 70 acts to protect cells against various cytotoxic agents and apoptotic stimuli thereby correlating with cellular resistance to apoptosis. Indeed, the overexpression of this protein in NS0 myeloma cells delayed apoptosis by 24 h and resulted in 2-fold increases in the number of resulting hybridoma fusions (Lasunskaia et al. 2005, 2003).

### Humanin

The pro-apoptotic Bax protein undergoes a conformational change in response to apoptotic signals that lead to its translocation from the cytoplasm to the mitochondrial membrane. Bax embeds itself into the mitochondrial membrane leading to permeation and the release of cytochrome *c*. A 24 amino acid peptide known as Humanin (HN) disrupts Bax translocation from the cytosol. The expression of heterologous humanin blocks particular cell death stimuli (Guo et al. 2003). However, apoptosis induced through Bax-independent pathways, such as TNF, are not affected by HN expression.

### Mitochondrial membrane potential

The permeability of the mitochondrial membrane determines the extent of stress required to lead to release of cytochrome *c* and other apoptotic proteins housed within the mitochondria. Greater durability of the membrane will require a higher stress threshold to disrupt cellular metabolism and lead to further downstream apoptotic events. Selecting cell lines that have higher mitochondrial membrane potential using rhodamine labeling represents another method to reduce apoptosis in fed-batch cultures. This strategy allows for the selection of cell lines that have a natural capacity for enhanced survival (Follstad et al. 2002).

## Regulating apoptosis to increase production

Expression of anti-apoptotic genes has had a significant effect on the survival of mammalian cells in culture. An important consideration is whether these genetic modifications have lasting effects on product titers. Several studies have already addressed this issue (Lasunskaia et al. 2003; Meents et al. 2002; Mercille et al. 1999; Tey et al. 2000a; Tey et al. 2000b) and more research is forthcoming. Their findings suggest that the expression of anti-apoptosis genes is most effective when cell survival limits productivity. In batch cultures, the expression of anti-apoptosis genes may not be particularly advantageous since viabilities remain high throughout the cell cultures. However, in fed-batch and perfusion cell culture experiments, the increased viabilities may significantly improve productivity in mammalian cells engineered to express anti-apoptosis genes. For example, hybridomas engineered to express E1B-19K showed a 40% increase in monoclonal antibody yield in perfusion culture due to a 2-fold increase in viable cell density (Mercille et al. 1999). Similarly, CHO cells engineered to express Bcl-2 exhibited a 40% increase in antibody titer in fed-batch cultures due to higher viable cell numbers and extended culture operating times (Tey et al. 2000a; b). Thus, the value of anti-apoptosis genes is likely to be greatest for those culture conditions in which the producer cells are exposed to significant external or internal stresses capable of activating the cell death cascade.

## Cell cycle arrest

An area that has recently gained much attention is gene expression levels and protein production throughout different phases of the cell cycle. Arrest in the G1 phase of the cell cycle can sometimes lead to more protein production, resulting in its designation as the production phase by some researchers (Mazur et al. 1998). Attempts to prolong the G1 phase have been a focus of several groups. By halting cell growth at the G1 phase using cytostatic genes such as cyclin dependent kinase (cdk) inhibitors p21 and p27, studies have shown increased productivities

(Mazur et al. 1998; Simpson et al. 1999). One such study employed CHO cells expressing a model product gene secreted alkaline phosphatase (SEAP). Control CHO cells were compared with CHO cells expressing p27 to find that growth arrest caused by the cdk inhibitor led to a 10 to 15-fold increase in SEAP production per cell (Mazur et al. 1998). Interestingly, the expression of bcl-2 family members not only delays the onset of apoptosis, but also increases the percentage of cells in the G1 phase (Mercille et al. 1999).

### Sodium butyrate

Various mammalian expression systems use promoters that can be stimulated with chemicals to remain highly active. For example, the addition of sodium butyrate, NaBu, in cell cultures has led to increases the expression of genes under the control of certain promoters. However, NaBu eventually causes growth arrest and apoptosis by inducing intracellular stress (Kim and Lee 2002a; Lee and Lee 2003). Cells designed to express monoclonal antibodies under the control of cytomegalovirus (CMV) or simian virus 40 (SV40) promoters show higher product yield with NaBu, although viabilities are compromised. To exploit the benefits of NaBu and lower its cytotoxic effects, different strategies to limit apoptosis have been combined with NaBu. In particular, Bcl-2 expression in combination with NaBu has led to increased antibody production with less cell death (Kim and Lee 2002a; Lee and Lee 2003). CHO cells not protected from the apoptotic effects of NaBu showed low viabilities in comparison with their Bcl-2 expressing counterparts. Monoclonal antibody yields were measured to find that CHO cells expressing Bcl-2 in the absence of NaBu produced only 10 $\mu g\ ml^{-1}$ of antibody, whereas the same cells in the presence of NaBu produced 51 $\mu g\ ml^{-1}$ of antibody (Lee and Lee 2003). Another strategy to limit apoptosis utilized the caspase-3 inhibitor Ac-DEVD-CHO which also delays NaBu-induced apoptosis in recombinant CHO cells producing humanized antibody but showed no improvement in antibody production. A concentration of 5 mM NaBu resulted in a culture viability of 65% in the presence of caspase inhibitor compared to a viability of only 25% in CHO cells cultured without the caspase inhibitor after a 72-h period (Kim and Lee 2002a). A better understanding of the role of anti-apoptosis factors on the cell cycle should lead to even better strategies to improve cellular productivities.

## Conclusions

A significant amount of biotechnological research has focused on improving bioreactor productivity through environmental manipulation such as supplementation of culture media or applying perfusion and fed-batch techniques. More recently, controlling signaling pathways has gained importance for developing more durable cell lines. Intracellular pathways for apoptosis may be regulated to improve cell performance and bioreactor productivity. The intracellular components of the apoptosis cascade are now being unraveled to reveal a wide array of cellular factors and complex pathways converging in programmed cell death. Cell engineering methodologies have shown that expression of cell fortifying proteins in combination with optimized bioreactor environments can further improve production levels for a number of biotherapeutics.

The use of anti-apoptotic Bcl-2 family members has been shown to increase viabilities in cell cultures producing monoclonal antibodies. The over-expression of these genes can help maintain mitochondrial integrity and inhibit the release of apoptosis instigating factors such as cytochrome *c*. Caspases inhibitors such as the Inhibition of Apoptosis Protein (IAP) family members, CrmA, and p35 have also been shown to be useful in the inhibition of apoptosis for particular culture insults. In addition, variants of the wild-type anti-apoptosis proteins can sometimes be even more effective in inhibiting the cell death pathway than native wild-type proteins. Other techniques such as RNA inhibition may also prove to be effective in limiting cell death in cell culture in the future. Approaches for limiting apoptosis should also consider cross-communication among the multiple cell death

pathways. Combinatorial methods that block cell death at multiple steps of a pathway or various pathways can be applied along with molecular biology approaches that provide improved activities for known anti-apoptosis genes expressed in mammalian cell lines. As we gain better insights into the molecular mechanisms behind this cascade, even better strategies will be devised for controlling the cell death response for animal cell cultures.

Similarly, our understanding of the relationship between environmental conditions and apoptosis has grown allowing us to ''fine-tune'' a culture environment that limits the activation of the cell death cascade. Anti-apoptosis strategies that consider both the external environment and the intracellular biology may lead to the greatest improvements in cell survival.

Future studies in the area of cellular engineering may also focus on combining anti-apoptosis genes with other genes that alter cell function in order to enhance cell durability upon exposure to particular stress-inducing environments. The alternation of cell cycle using genes or environmental manipulation is another area that will be important in improving cellular production of recombinant proteins in mammalian cell cultures. These cell engineering methods should lead to even more valuable mammalian cell lines for biotechnologists in the future. The application of such cell lines in combination with improved nutrient formulations and efficient bioreactor configurations will lead to even better bioreactor performance and enhanced product yields in the coming years.

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