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Life and death in mammalian cell culture: strategies for apoptosis inhibition

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Mammalian cell culture is widely used to produce valuable biotherapeutics including monoclonal antibodies, vaccines and growth factors. Industrial cell lines such as Chinese hamster ovary (CHO), mouse myeloma (NS0), baby hamster kidney (BHK) and human embryonic kidney (HEK)-293 retain many molecular components of the apoptosis cascade. Consequently, these cells often undergo programmed cell death upon exposure to stresses encountered in bioreactors. The implementation of strategies to control apoptosis and enhance culture productivities represents a major goal of biotechnologists. Fortunately, previous research has uncovered many intracellular proteins involved in activating and inhibiting apoptosis. Here, we summarize three apoptotic pathways and discuss different environmental and genetic methodologies implemented to limit cell death for biotechnology applications.

Optimization of mammalian cell culture technology is essential for the economical production of biopharmaceuticals, such as monoclonal antibodies, biotherapeutics and vaccines in bioreactors. A major problem faced in bioreactor culture is cell death, which decreases overall biopharmaceutical yield. Cell death in bioreactors has various causes, including mechanical agitation, nutrient depletion, waste byproduct accumulation, hypoxia and viral infections. Cell death occurs in two general forms: necrosis and apoptosis. Necrosis results from immediate, extreme conditions that physically damage cells causing them to swell and rupture, releasing their cellular contents into the surrounding environment. Apoptosis, however, also referred to as programmed cell death, is a regulated physiological response resulting from a non-lethal stimulus that activates a cellular cascade of events culminating in cell death. To optimize cell viabilities and protein yields in culture, attention is now being devoted to strategies for controlling cell death. This review will examine new methods being applied for limiting cell death in biotechnology, after an overview of the apoptosis pathways.

Cell death by apoptosis

Apoptosis is a genetically controlled process and is morphologically recognized by cell and chromatin shrinkage followed by plasma membrane blebbing. Blebbing involves the shedding of membrane fragments from the cell in the form of apoptotic bodies that often include cytosolic and nuclear contents. An apoptotic Chinese hamster ovary (CHO) cell exhibiting membrane blebbing and chromatin shrinkage is compared to a wild-type CHO cell following staining with acridine orange and ethidium bromide (Figure 1). These apoptotic bodies can be phagocytosed *in vivo* although *in vitro* they might 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.

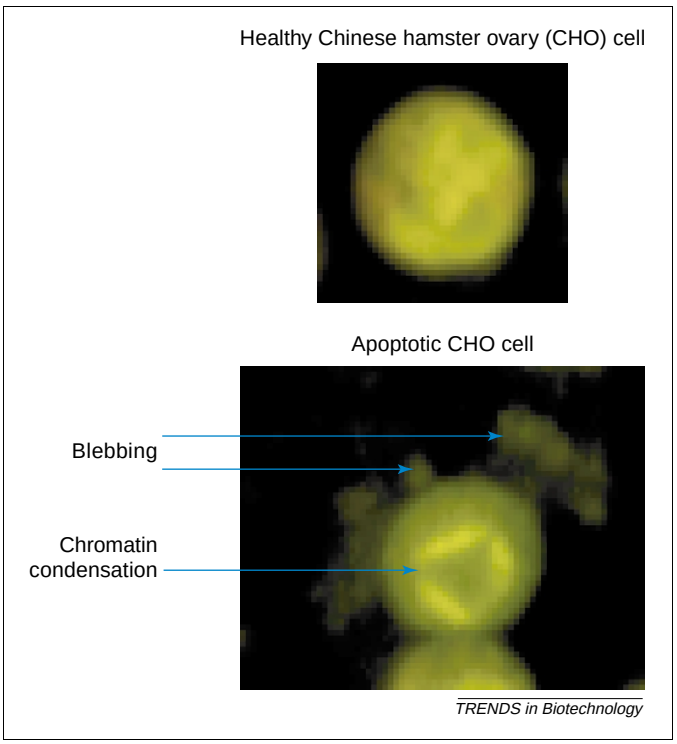

Figure 1. Healthy and apoptotic chinese hamster ovary (CHO) cells stained with acridine orange and ethidium bromide. The top photograph shows a healthy CHO cell, which is represented here as morphologically spherical with its cell membrane intact. The bottom photograph shows a CHO cell undergoing apoptosis after exposure to stress, in which the membrane is no longer intact but shedding apoptotic bodies. The process by which membrane fragments shed from the cell body is referred to as blebbing.

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Although the apoptotic program is highly complex and the cellular events involve the activation of many signaling cascades resulting from either external stress signals or internal organelle-specific initiation events, three main pathways predominate: (i) the mitochondrial-mediated pathway; (ii) the endoplasmic reticulum (ER) stress-induced pathway and (iii) the cell surface-mediated signal transduction pathway (Figure 2). Many apoptosis signals converge on the mitochondria – the energy generators for the cell, which also, perhaps not coincidentally, house numerous apoptosis-instigating molecules, including cytochrome c, second mitochondrial activator of caspases (SMAC)/DIABLO [direct inhibition of apoptosis protein (IAP) binding protein with low pI] and apoptosis inducing factor (AIF). Maintaining the mitochondrial membrane potential (MMP) is an essential checkpoint in preventing the activation of the apoptosis pathway [1]. The bcl-2 family of proteins are prominent members of the apoptosis cascade operating at the outer mitochondrial membrane and perhaps other cellular membranes as well. The bcl-2 family consists of both pro- and anti-apoptotic members grouped into three families: (i) anti-apoptotic bcl-2 homologs containing Bcl-2 homology (BH) domains 1, 2, 3 and 4; (ii) pro-apoptotic members containing BH1, 2, and 3 homology domains; and (iii) BH3 only pro-apoptotic members (Figure 3) [2]. Upon exposure to intrinsic or extrinsic death signals, pro-apoptotic proteins such as Bax and Bak undergo structural modifications in which they alter the mitochondrial membrane integrity, causing the release of cytochrome c and other pro-apoptotic molecules [3–6]. These molecules are released in response to apoptotic stimuli, such as toxin exposure or DNA damage to promote activation of pro-caspase-9, a principal cysteine–asparate protease responsible for initiating cellular apoptosis signaling. Caspase-9, as well as the other caspases, exists in an inactive zymogen pro-caspase state that is activated through proteolytic processing. Activation of caspase-9 occurs in the apoptosome and involves apoptosis protein activating factor-1 (Apaf-1) and pro-caspase-9 in the presence of cytochrome c and dATP [7].

Opposing these pro-apoptotic signals are the anti-apoptosis proteins, including Bcl-2 and Bcl-x_L, which inhibit the release of pro-apoptotic molecules from the mitochondria by multiple mechanisms, including maintenance of mitochondrial membrane integrity and binding to pro-apoptosis members of the bcl-2 family. Metabolites can permeate the mitochondrial membrane through the voltage-dependent anion channel (VDAC), which seems to be regulated by the bcl-2 family proteins [2].

An ER stress-induced apoptotic pathway was found to be initiated by various conditions including the inhibition of protein glycosylation, reduced disulfide bond formation,

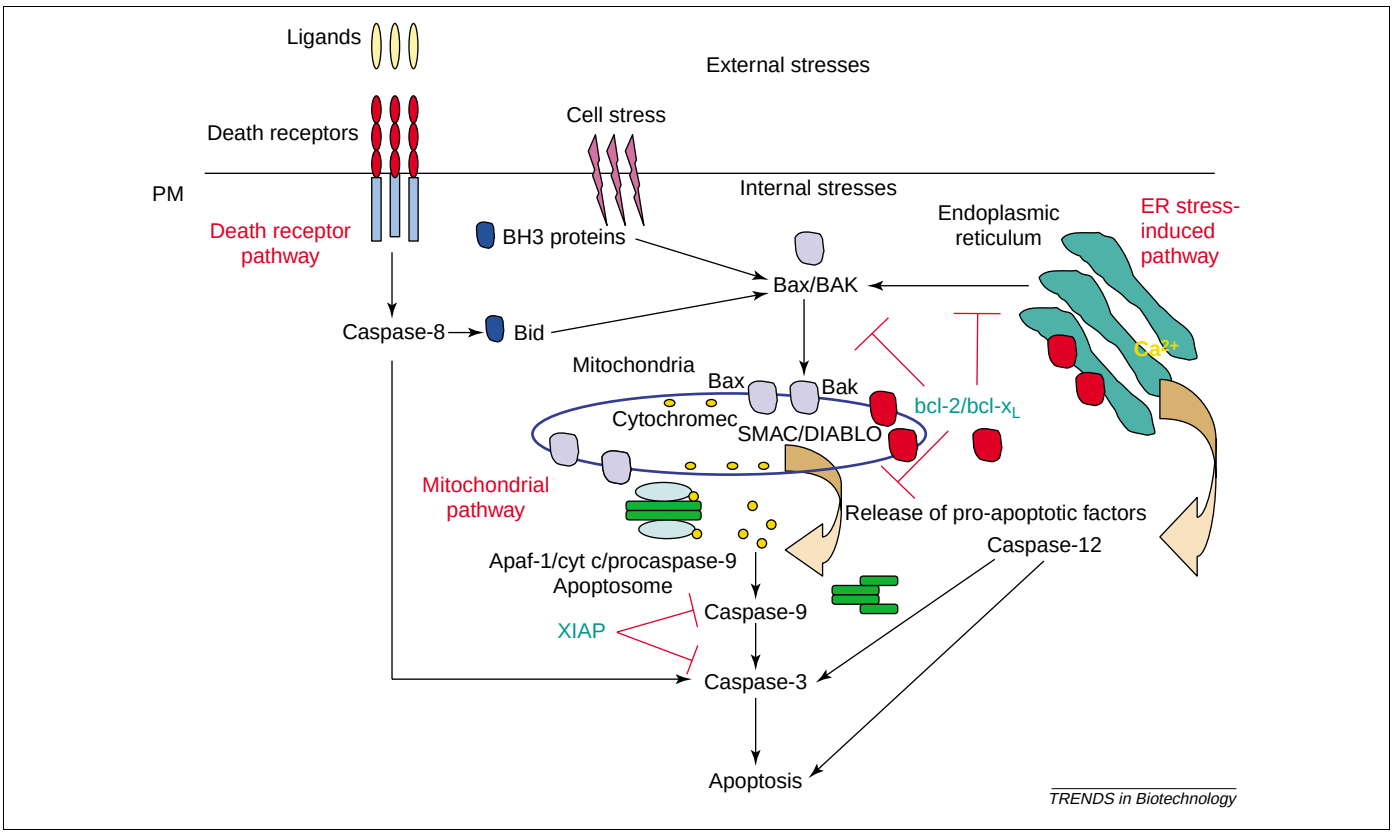

Figure 2. Molecular pathways leading to apoptosis. This schematic shows the three main pathways that can be activated when the cell encounters specific stresses. The cell-surface mediated or death receptor pathway includes the binding of a ligand to the TNF family of receptors and is generally activated by external stimuli. Mitochondrial apoptosis is induced by many intracellular stresses upregulating Bax and Bak levels. These proteins then translocate to the mitochondrial membrane leading to its permeability and the release of apoptotic proteins. The endoplasmic reticulum (ER) pathway is induced by misfolded and aggregated proteins and other stresses in the ER that leads to the release of Ca^{2+} and activation of the mitochondrial apoptosis pathway and independent ER-associated caspases. Bid is a member of the pro-apoptotic BH3 subfamily. Bax and Bak are both pro-apoptotic proteins containing multiple bcl-2 homology domains. SMAC, DIABLO, Apaf-1 and cyt c are among the apoptotic proteins housed in the mitochondria and are released upon loss of mitochondrial membrane integrity. XIAP is a member of the inhibitor of apoptosis protein family, which inhibits activation of downstream caspases. Abbreviations: Apaf-1, apoptosis protein activating factor-1; cyt c, cytochrome c; DIABLO, direct inhibition of apoptosis protein (IAP) binding protein with low pI; SMAC, second mitochondrial activator of caspases; XIAP, X-linked inhibitor of apoptosis.

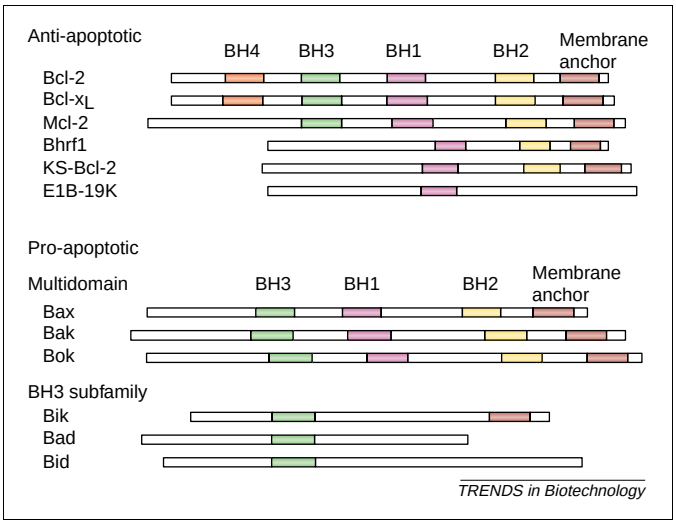

Figure 3. Bcl-2 family members including anti-apoptotic and pro-apoptotic members. Homology domains within the family can be seen. The primary anti-apoptotic members, Bcl-2 and Bcl-x_L, contain four bcl-2 homology domains (BH1 to 4). The pro-apoptotic family members include a group with BH1–3 homology domains and members with only BH3 domains.

calcium depletion from the ER lumen and defects in protein expression, folding or transport from the ER to Golgi [8]. Malfunction in the ER can activate ER stress transducer proteins that can, in some cases, lead to apoptosis through pathways that are dependent on or independent of the mitochondrial membrane transition. In mitochondrial-dependent paths, the ER senses local stresses through chaperones, Ca^{2+} binding proteins and Ca^{2+} release channels, which can relay ER Ca^{2+} responses to the mitochondria – a major commitment point for cell death or survival. Independent of mitochondrial activity, ER dysfunction can lead to apoptosis through pathways involving caspase-12 activation. For example, the ER stress response can lead to Bax and Bak translocation to the ER membrane causing ER calcium depletion, caspase-12 activation and ultimately apoptosis [9]. The ER also contains Bcl-2 anti-apoptosis proteins, suggesting that these family members might exert cytoprotective effects within the ER [10].

The receptor-mediated signal transduction pathway is triggered by the binding of ligands to death receptors at the cell plasma membrane. These receptors belong to the tumor necrosis factor (TNF) superfamily, which includes CD95/Fas and Apo2L/TRAIL [11]. Ligand binding to these receptors promotes the recruitment of the protein Fas-associated death domain (FADD) onto the cytoplasmic tail of the receptors. Bound FADD then recruits pro-caspase-8 through its death effector domain (DED), promoting dimerization and autocatalytic activation of this initiator caspase. The mitochondrial-mediated and cell surface signal-transduction pathways converge at the activation of caspase-3 (Figure 2). Thus, at the apex of the apoptosis cascades are the initiator caspases (caspase-8 in the cell surface-signaling pathway, caspase-9 in the mitochondrial-mediated pathway and caspase-12 in the ER pathway). The initiator caspases-8, -9, and -12 in turn activate the downstream effector class of caspases-3, -6, and -7. The activation of these effector caspases leads to the final execution of the cell death program. The receptor-mediated and mitochondrial pathways can also interact through the activation of Bid by caspase-8 leading to Bax and Bak structural modification and permeabilization of the mitochondrial membrane.

Apoptotic detection

Assays to detect apoptosis in cell populations include measuring DNA fragmentation with DNA ladders, a signature of apoptosis, or detecting activation of apoptosis-induced proteases, such as caspase-3 or poly(ADP ribose) polymerase (PARP) using Western blot techniques. Methods for detecting apoptosis in individual cells include terminal deoxynucleotidyl transferase nick-end-labeling (TUNEL), annexin V binding to cell membranes and DNA staining with propidium iodide, 4,6-diamino-2-phenylindole (DAPI), or ethidium bromide.

Flow cytometry is based on laser technology and has become a valuable tool for detecting 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: (i) light scattering based on cell size and shape; (ii) propidium iodide exclusion of cells; (iii) analysis of organelle function (e.g. mitochondria with rhodamine and lysosomes with acridine orange); (iv) end labeling DNA strand breaks using TUNEL or terminal deoxynucleotideyl transferase (TdT) and biotinylated dUTP, or (v) 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. However, the distinction between necrosis and apoptosis might not always be clear for some stains. The appropriate apoptosis assay chosen will depend on the experience and facilities of the individual researcher. However, biotechnologists will favor methods that allow early detection and adequate quantification of apoptosis in cells.

Methods for apoptosis inhibition

Given that the induction of apoptosis can lead to the loss of viable cells in bioreactors, several methods are being evaluated to limit the activation of the apoptosis cascade. Inhibiting or slowing the onset of cell death is beneficial because extending cell lifetimes can lead to more-productive cell culture systems for biotechnology applications [12]. Two strategies being examined for enhancing cell survival in bioreactors are the manipulation of the external environment through media supplementation (including nutritional or chemical methods) and alteration of intracellular biochemistry using genetic engineering approaches (Table 1).

Media supplementation

Given that the onset of apoptosis is often triggered by conditions outside cells, one strategy to limit cell death is to alter the extracellular environment. Altering the media can be a highly effective technique in prolonging cell

Table 1. Compilation of representative methods of cellular protection from apoptosis for specific cell types[a]. Multiple cell types are shown to have increased viability through the use of one or several protective methods

Cell line	Method of protection	Refs
NS0, CHO, HEK-293, BHK, Hybridoma	Bcl-2	[25–27,29,32–36,39]
CHO, HEK-293	Bcl-2Δ mutant	[39]
CHO, BHK, Hybridoma	Bcl-x_L	[28–30,32,40]
CHO	Bcl-x_LΔ mutant	[40]
NS0	E1B-19K	[45]
Hybridoma	Bhfr-1, kbcl-2	[31]
CHO, HEK-293	XIAP/XIAP mutant	[22,38]
CHO, HEK-293	CrmA/CrmA mutant	[22,38]
CHO, HEK-293	Caspase inhibitors: Z-IETD-fmk, Z-LEHD-fmk, Z-VAD-fmk	[22]
Hybridoma	Caspase inhibitors: Ac-DEVD-cho, Z-VAD-fmk	[20]
Human epidermal keratinocytes	Dominant negative caspase-9	[42]
Jurkat cells	Dominant negative FADD	[43]
CSM14.1 neuron cells, SF268 glioblastoma cells, HCT116 colon cancer cells, PC-3 prostate cancer cells	Humanin	[47]
CHO	Transferrin and insulin-like growth factor-I receptor	[14]
NS0	Hsp70	[46]
Hybridoma	High mitochondrial membrane potential selection	[48]
CHO	Suramin	[13]
HeLa	Silkworm hemolymph	[19]
Hybridoma	Rapamycin	[24]
Hybridoma	Serum growth factors	[34]
NS0, Hybridoma	Glutamine	[18,28]
VERO	Galactose, glutamine	[15]
CHO	Glutamine, asparagine, glucose feeding	[16]
CHO, Hybridoma	Glycine betaine, glycine, asparagines, threonine	[17]

[a]Abbreviations: Ac-DEVD-cho, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde; BHK, baby hamster kidney; CHO, Chinese hamster ovary, CSM, crude synaptic membrane; FADD, Fas-associated death domain; HEK-293, human embryonic kidney 293; NSO, NSO mouse myeloma cells; VERO, African green monkey (VERO) cells; Z-IETD-fmk, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone; Z-LEHD-fmk, benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone; Z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

viability in culture through the addition of nutrients or supplementation with anti-apoptotic chemicals or peptides. Serum is often an effective anti-apoptosis agent during all stages of cell growth following nutrient depletion. However, the growth of cultures in serum-free medium is often a requirement for current industrial mammalian cell cultures. As a result, selective agents have been considered as potential additives for preventing apoptosis in a serum-free environment. Suramin, a polysulfated naphthylurea, protects CHO cells in serum-free culture from apoptosis during the exponential growth phase. Unlike serum, suramin is unable to protect cells from death-phase apoptosis, indicating that unknown anti-apoptotic components are present within serum [13]. Two other agents that have been considered in efforts to suppress cell death in serum-free environments are insulin and transferrin. Transferrin has a role in transporting iron, an essential element for cell growth. Insulin-like growth factor-I (IGF-I) can replace insulin as a mitogen and inhibit apoptosis in cultures exposed to death-inducing stimuli through the IGF-I receptor. Cell lines overexpressing the IGF-I receptor in combination with IGF-I and transferrin added to the medium have higher viabilities and maintain growth of CHO cells in serum-free media [14].

Nutrient feeding of cell culture media is another effective method for preventing apoptosis. High-density mammalian cell cultures can be deprived or fed specific nutrients to determine how apoptosis might be induced or inhibited. Apoptosis was induced in the absence of glutamine and galactose for African green monkey VERO cells and CHO cells. Studies show that feeding VERO cells with these same nutrients protected them from apoptosis. As a result, nutrient feeding can provide better protection of cell cultures [15,16]. Elevated concentrations of some amino acids protect cells from diverse environmental stresses, such as elevated partial pressure of CO_2, hyperosmolarity and nutrient starvation. Addition of either glycine betaine, glycine, asparagine, or threonine to hybridoma and CHO cells restored growth of cells exposed to these stresses [17]. Studies have also shown that an increase in apoptosis is paralleled by an increase in the expression of the growth arrest- and DNA damage-inducible gene 153 (*gadd153*) in NS0 myeloma cells. The suppression of *gadd153* expression through the addition of nutrients, particularly glutamine, led to a delay in the onset of apoptosis [18]. The addition of silkworm hemolymph also inhibits apoptosis in mammalian cell culture systems, perhaps by decreasing cell detachment from an adhering surface [19].

Another approach for apoptosis inhibition is to supplement the media with additives that block events within the cell-death cascade. In particular, the caspases have a central role in the activation of apoptosis through their proteolysis of various key intracellular proteins. The addition of the caspase inhibitors *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-cho) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) suppresses the apoptotic program in hybridoma cells deprived of glutamine [20]. In addition, the use of combinations of these caspase inhibitors prolongs cell viability in cultures under nutrient depletion conditions [21]. Different stimuli might activate different apoptosis cascades and thus the desired inhibitor might differ for a given stimulus. Spent

medium-induced death was delayed more readily with the caspase-8 inhibitor, benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (Z-IETD-fmk); whereas etoposide-induced death was stalled more readily with the caspase-9 inhibitor, benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone (Z-LEHD-fmk) [22]. Caspase inhibition however, does not appear to provide complete cytoprotection from the apoptotic process because mitochondrial dysfunction, phosphatidylserine exposure, plasma membrane permeabilization and loss of clonogenic potential are still observed [23]. As an alternative, the addition of rapamycin to culture medium has been effective in limiting apoptosis in addition to arresting G1-phase [24]. Hybridoma cell-death in batch culture was delayed 48 hours and recombinant antibody titer increased more than twofold following the addition of this chemical agent.

Genetic strategies

Recent genetic strategies have proven successful in delaying apoptosis in cell culture. Several viral and cellular proteins inhibit apoptosis in cells at distinct points along the apoptotic pathways, and the expression of genes encoding these proteins can often modify the cell-death response in mammalian cell cultures.

Bcl-2 and Bcl-x_L are prominent anti-apoptotic proteins that inhibit the release of pro-apoptotic molecules from the mitochondria. NS0, CHO, BHK and hybridoma cells transfected with *bcl-2* have higher viabilities and improved robustness compared with control cells when deprived of serum, glucose, glutamate, asparagine and other nutrients and following exposure to toxins, viral infections or other adverse conditions [25–35]. The expression of *bcl-x_L* is also an effective method for inhibition of cell death in CHO and BHK cells [32,33,36].

Caspases have also been inhibited using genetic strategies that interfere with their activation. The X-linked inhibitor of apoptosis (XIAP) is an inhibitor of caspases-9, -3 and -7 that acts downstream from Bcl-2 and Bcl-x_L in the apoptosis pathway. The expression of recombinant XIAP has been shown to increase viabilities of CHO and human embryonic kidney (HEK) 293 cells in culture [22]. The cytokine response modifier CrmA, which inhibits caspase-8, can also be expressed to prolong cell viability [21,22,37].

Variants of three anti-apoptosis proteins (Bcl-2, Bcl-x_L and XIAP) have been generated and compared with their wild-type counterparts in engineered BHK, CHO and HEK-293 cell lines [38–40]. The mutations in these genes were generated in several ways. For the Bcl-2 and Bcl-x_L variants (Bcl2Δ and Bcl-x_LΔ) a region of the protein containing a nonstructured loop between the BH3 and BH4 domains was deleted [41]. This region of the protein is processed by caspases during the apoptotic cascade leading to degradation of Bcl-2 and its conversion from an anti-apoptotic protein to a pro-apoptotic protein. CHO and BHK cells expressing the Bcl2Δ variant protein survive for longer periods than cells expressing the wild-type Bcl-2 protein following exposure to multiple insults, including serum withdrawal and Sindbis virus infection. Unlike the Bcl2Δ variant, clonal isolates of CHO-*bcl-x_LΔ* expressed the mutant Bcl-x_LΔ protein at much lower levels than the wild-type protein [40]. Confocal microscopy suggested that the Bcl-x_LΔ protein is less stable than the wild-type protein because of the tendency of the mutant protein to aggregate in mammalian cells. Nonetheless, cells expressing Bcl-x_LΔ provided equivalent levels of protection and also enhanced proliferation of CHO cells under particular apoptosis-inducing stimuli.

For XIAP, deletion mutants were created by expressing either the N-terminal baculovirus IAP repeat (BIR) domains or the C-terminal RING domain. The RING domain was found to possess pro-apoptotic activity and when removed from the XIAP protein, the mutant protein including the BIR domains inhibited apoptosis as well or better than the full-length XIAP in cell cultures [22,38]. This enhanced protection by the BIR domain mutant might have resulted from improved stability and lowered aggregation of the protein because of the removal of the RING domain.

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 [42]. A similar approach can be applied for other initiator caspases as well as pro-apoptotic proteins such as FADD [43].

Several viral proteins from adenovirus, human immunodeficiency virus-1, Karposi's sarcoma-associated herpesvirus, human T-cell leukemia virus-1, hepatitis B virus, and Epstein Barr virus keep mitochondrial membrane permeability intact and target 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 [44]. With the goal of producing more robust hybridoma cell lines, viral *bcl-2* homologues *ksbcl-2* from Karposi's sarcoma-associated herpesvirus and *bhrf-1* from Epstein-Barr virus were used in combination with apoptotic stimuli to examine the effectiveness of these proteins. Cells expressing Bhrf-1 afforded the best protection against apoptosis induced in glutamine-free culture conditions [30]. Previously, the E1B19K adenoviral gene had also been shown to provide protection against apoptosis for NS0 cells exposed to apoptotic stimuli [45].

Heat shock proteins of the hsp70 family function as molecular chaperones involved in protein folding, transport and degradation. In addition, Hsp 70 protects cells against various cytotoxic agents and apoptotic stimuli thereby providing cellular resistance to apoptosis. Indeed, the overexpression of this protein in NS0 myeloma cells delayed apoptosis by 24 hours and resulted in twofold increases in the number of resulting hybridoma fusions [46].

Bax is a pro-apoptotic protein that undergoes a conformational change in response to signals that lead to its translocation from the cytosol to the mitochondria and the release of cytochrome c. Humanin (HN) is a 24 amino acid peptide that interferes with Bax translocation from

the cytosol and the expression of heterologous humanin blocks particular cell-death stimuli [47]. However, apoptosis induced via TNF, a Bax-independent pathway, is not affected by HN expression.

Selecting cell lines that have higher mitochondrial membrane potential using rhodamine labeling is another method for reducing apoptosis in fed-batch cultures. This strategy allows the selection of cell lines with a natural capacity for improved survival [48].

Clearly, the expression of these anti-apoptosis genes has had a significant effect on the survival of mammalian cells in culture. One important question is whether these genetic modifications have any effect on the final product titers. Several studies have already addressed this issue [25,26,29,46,49] and further research is forthcoming. The findings suggest that the expression of anti-apoptosis genes is most effective when cell survival limits productivity. In short-term batch cultures, the expression of anti-apoptosis genes might not be particularly advantageous because viabilities remain high throughout the cell culture. However, in extended fed-batch and perfusion cell-culture experiments, the increase in viabilities might be significant for 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 because of a twofold increase in viable-cell density [49]. Similarly, CHO cells engineered to express *bcl-2* exhibited a 40% increase in antibody titer in fed-batch cultures because of higher viable-cell numbers and extended culture operating times [25,26]. 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.

Conclusions and future work

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. As we gain better insights into the molecular mechanisms behind this cascade, better strategies will be devised for controlling the cell-death response for animal cells in culture. For example, conditions in the ER are now recognized to have an important role in the onset of apoptosis for some stimuli, and this pathway represents a valid target for anti-apoptosis methods. In addition, several anti-apoptosis genes have been discovered recently in viruses and cells and these represent exciting new opportunities for limiting cell death. Of course, all approaches for limiting apoptosis must be applied with consideration for the multiple cell death pathways that exist and the interconnectivity that links these cascades. Combinatorial methods that block cell death at multiple steps or in multiple pathways can be applied along with molecular biology approaches that provide improved activities for known anti-apoptosis genes inserted into mammalian cell lines. Similarly, our understanding of the relationship between environmental conditions and apoptosis has grown and this knowledge will allow us to define 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 should lead to the greatest improvements in cell survival; providing unequalled bioreactor performance and enhanced biopharmaceutical productivities in the future.

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