# Anti-apoptosis Engineering

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**Abstract** An increased understanding of apoptosis makes anti-apoptosis engineering possible, which is an approach used to inhibit apoptosis for the purpose of therapeutic, or industrial applications in the treatment of the diseases associated with increased apoptosis, or to improve the productivity of animal cell cultures, respectively. Some known anti-apoptotic proteins are the Bcl-2 family, IAP (inhibitor of apoptosis) and Hsps (heat shock proteins), with which anti-apoptosis engineering has progressed. This article reviews anti-apoptosis engineering using known anti-apoptotic compounds, and introduces a 30 K protein, isolated from silkworm hemolymph, as a novel anti-apoptotic protein, that shows no homology with other known anti-apoptotic proteins. The regulation of apoptosis, using anti-apoptotic proteins and genes originating from the silkworm, *Bombyx mori*, may provide a new strategy in this field.

*Keywords*: apoptosis, anti-apoptosis engineering, Bcl-2 family, IAP, Hsps, 30K protein

## INTRODUCTION

### Overview of Apoptosis

Apoptosis, or programmed cell death, is a physiological cell death, which is morphologically distinguishable from the passive cell death mechanism known as necrosis [1, 2]. As shown in Fig. 1, apoptosis is accompanied by chromatin condensation, cell shrinkage, membrane blebbing, apoptotic body formation and DNA fragmentation [3], while necrosis is typified by cell swelling, mild clamping of chromatin and lysis, which results in the release of the cell constituents. Programmed cell death occurs in most animal tissues to eliminate damaged, harmful cells, or those that are produced in excess, and this is mediated by apoptosis [4,5].

The concept of programmed cell death has its roots in embryological and pathological research. In the 1950's, Glucksmann theorized that cell death was vital to the proper developmental plan of vertebrates [6]. Also, pathological investigations have long described the cellular demise associated with nuclear 'pyknosis', which indicates a characteristic shrinkage and condensation of the nucleus. Kerr and coworkers provided the convenient name, 'apoptosis', for this process [7]. The wide acceptance of this term, originally used to describe morphological features of programmed cell death, has coincided with the elucidation of a number of molecular events involved in programmed cell death [8].

Genetic studies of the nematode, *Caenorhabditis elegans*, have led to the identification of three genes, *ced-3*, *ced-4* and *ced-9*, which are related with apoptotic cell death. Two of these, *ced-3* and *ced-4*, are required for the apoptotic program [9]. *Ced-9*, which functions upstream of the *ced-3* and *ced-4*, negatively regulates the apoptotic program by preventing activation of the *ced-3* and *ced-4* [10]. *Bcl-2*, the mammalian homologue of *ced-9*, has protective effects from apoptosis [11], which suggests the molecular mechanisms of programmed cell death have been conserved in the progression from nematodes to mammals. The *ced-3* gene encodes a cystein protease, and ICE (interleukin-1β-converting enzyme) has been reported as a mammalian homologue of the Ced-3 [12]. It has also been reported that the Ced-4 plays a central role in the cell death mechanism linking the Ced-9 and Bcl-2 family to the Ced-3 and ICE family [13]. It has been discovered that Apaf-1 is a mammalian homologue

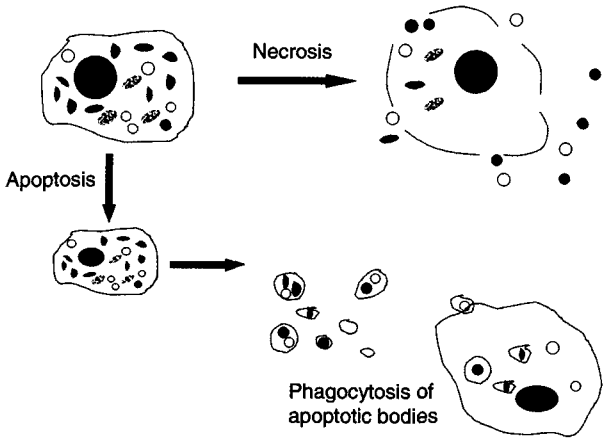


**Fig. 1.** Cell death can occur by either apoptosis or necrosis.

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of the Ced-4, and the Ced-4 has been suggested to trigger the activation of caspase 3 by binding of the cytochrome c released from mitochondria [14].

### Signaling Pathway Leading to Apoptosis

The caspases, derived from ICE/Ced-3 family members, play a critical role in the execution of apoptosis, and are responsible for many of the associated biochemical and morphological changes. The 'c' in caspase denotes a cystein protease, and the 'aspase' refers to the ability of this enzyme to cleave after an aspartic acid residue. The observation that ectopic overexpression of many caspases resulted in apoptosis indicates a role of caspases in apoptosis. Further evidence supporting this role is the apoptosis inhibiting ability of specific protease inhibitors, including the cowpox viral serpin CrmA [15] and baculovirus P35 [16]. Caspases are synthesized as inactive proenzymes, which are activated by cleavage at aspartic acid residues to mature the enzymes. The initiator caspases, such as caspase 2, 10 and 8, activate themselves due to autocatalysis in response to an apoptotic stimuli, and then directly activate effector caspases, such as caspase 3, 6 and 7 [17].

The signaling pathway leading to apoptosis can be conveniently subdivided into four phases: initiation, signaling, effector and degradation [18]. Once apoptosis has been initiated by an inducer, such as chemicals (etoposide, staurosporine) or cell surface receptors (Fas receptor, TNF receptor), the apoptotic signals are transmitted through the cellular machinery. This signaling phase results on the activation of caspases, which execute the apoptosis in the effector phase. Sequentially activated caspases are responsible for the biochemical changes observed in apoptosis, such as degradation of DNA and cellular substrates. We describe the two major apoptotic signaling pathways; the mitochondrial and cell death receptor pathways.

The mitochondria are considered major players in the apoptosis of mammalian cells, and undergo functional and structural changes during the death process. Permeability transition pore opening caused by death stimuli leads to the release of the mitochondrial apoptotic factors, AIF (Apoptosis-inducing factor) and cytochrome c [19]. As shown in Fig. 2, Apaf-1 binds to procaspase-9 in the presence of dATP and cytochrome c, the interaction of which is mediated by the caspase recruitment domains (CARDs) found on these two proteins [20]. The complex formation between Apaf-1 and procaspase-9 leads to the cleavage and activation of caspase-9 by self-proteolysis, and in turn the caspase-9 directly activates the effector caspase-3 and -7 [21].

The death-signaling pathway emerging from aggregation of the cell surface receptor (CD95/APO-1/Fas) has been most studied [22]. With the Fas-induced apoptosis, stimulation by its natural ligand, or by agonist antibodies, leads to the formation of a death-inducing signaling complex (DISC). The DISC contains FADD/MORT-1, a Fas-associated death domain adapter protein that binds to the receptor through its C-terminal death

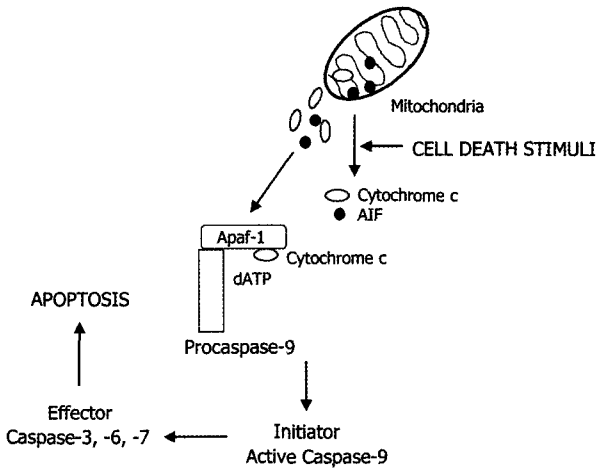


**Fig. 2.** Caspase activation via the mitochondrial pathway.

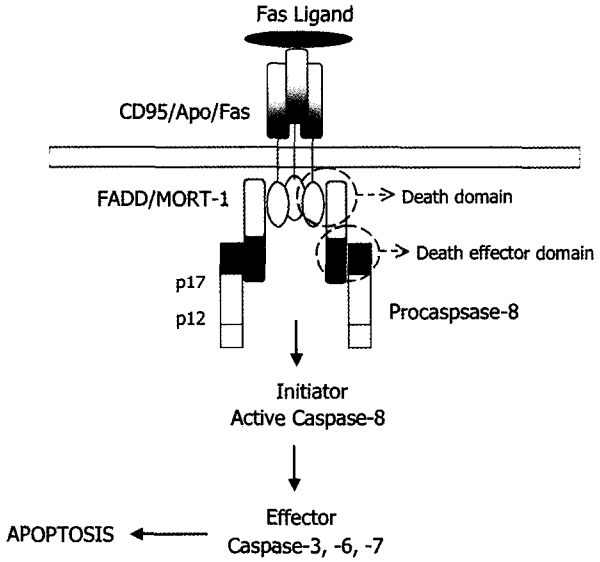


**Fig. 3.** Caspase activation via the CD-95/APO-1/FAS cell death receptor.

domain (DD). The FADD/MORT-1 also contains an N-terminal death effector domain (DED), which recruits procaspase-8 or -10 through a DED-DED interaction (Fig. 3). Once procaspase-8 has been recruited by the DISC, it is proteolytically processed into an active protease containing p17 and p12 subunits that are released from the DISC [23]. The active caspase-8 in turn can process other downstream caspases, such as caspase-3 [24].

## ANTI-APOPTOSIS ENGINEERING

### Anti-apoptotic Proteins

*Bcl-2*, the mammalian homologue of *ced-9*, which

prevents programmed cell death in *C. elegans,* has a protective effect against apoptosis [11]. It was originally discovered in follicular lymphomas due to its involvement in the t (14; 18) chromosomal translocation, where the *bcl-2* gene is translocated into the immunoglobulin heavy chain locus, resulting in its massive overexpression in B cells [25]. It is evident that the Bcl-2 is a general anti-apoptotic protein capable of protecting cells from a wide variety of apoptotic stimuli, including growth factor withdrawal, irradiation, cytotoxic drugs, heat, monocytes and the deregulated oncogenes [26]. The inhibition mechanism of the Bcl-2 controls the release of cytochrome c from the mitochondria by regulating the permeability of transition pore. Bcl-xL, Mcl-1, A1 and Boo have been identified as members of the Bcl-2 family, since they are closely related to Bcl-2. It is interesting that the Bcl-2 can also inhibit apoptosis downstream from the release of cytochrome c, suggesting another possible anti-apoptotic mechanism [23].

The IAP (Inhibitor of apoptosis) family plays an evolutionarily conserved role in the regulation of programmed cell death, with the first members of the family found in baculovirus as a result of their ability to attenuate the apoptosis induced by viral infections [27, 28]. To date five mammalian IAP homologues (NAIP, cIAP/HIAP2. cIAP2/HIAP1, hILP/XIAP, and survivin) have been cloned [29]. They have all been reported to inhibit apoptosis, and function as direct inhibitors of the activated effector caspases, caspase-3 and caspase-7 [30-32]. The ability of cIAP1 and cIAP2 to associate with TNF-receptor associated factors (TRAFs) suggests that they may also inhibit the proteolytic processing of caspases at the receptor complex [33,34]. Furthermore, cIAP, cIAP2 and XIAP, are also able to inhibit the cytochrome c-induced activation of caspase-9 [32].

All cells, both prokaryotic and eukaryotic, respond to the elevation of temperature accompanied by the synthesis of proteins, known as heat shock proteins (Hsps, stress proteins). Hsps are among the most conserved proteins known, with respect to both their structure and function as molecular chaperons, in phylogeny [35-38]. The overexpression of Hsp 70 or Hsp 27 has been demonstrated to make cells resistant to death. The overexpression is induced, not only by heat, but also by most apoptotic stimuli, including TNF, monocytes, UV radiation, oxidative stress, ceramide and several chemotherapeutic drugs [39,40]. Hsp 27 may also neutralize the toxic effect and inhibit the formation, of reactive oxygen species (ROS) [41]. Modulation of apoptosis by the Bcl-2 family, IAPs and Hsps, is schematically presented in Fig. 4.

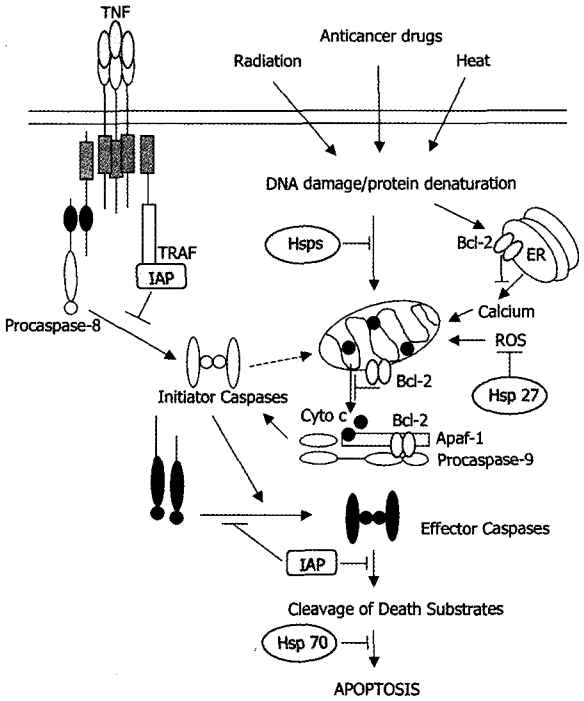


**Fig. 4.** Modulation of apoptosis by Bcl-2 family, IAPs and Hsps.

## Therapeutic and Industrial Aspect of Anti-apoptosis Engineering

Since apoptosis is essential to embryogenesis, development of the immune system and the maintenance of tissue homeostasis [5], it is not surprising that dysregulation of apoptosis is the direct cause of a number of human diseases [42]. Excessive cell death, due to increased apoptosis, results in neurodegenerative and autoimmune disorders, such as insulin-dependent diabetes mellitus and AIDS (acquired immunodeficiency syndrome). The accumulated cells as a result of cancer, or autoimmune diseases, such as systemic lupus erythematous and viral infections, are involved in the inhibition of apoptosis in response to appropriate stimuli [43].

Many researchers have investigated the inhibition of neural cell death by the introduction of anti-apoptotic genes, such as *bcl-2,* which could possibility lead to a therapeutic approach for the treatment of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases. The cowpox virus encodes a protein Crm A, which is known to be a caspase inhibitor [44]. It was reported that CrmA cDNA cloned into chicken neuronal cells protected from the growth factor withdrawal-induced apoptosis [45]. The expression of baculovirus caspase inhibitor P35 in neurons prevents the apoptosis induced by various agents in different neuronal populations [46]. Inhibition of apoptosis by the P35 expression in oligodendrocyte alleviated the severity of the neurological manifestation observed in autoimmune demyelinating diseases [47]. Anti-angiogenesis using IAP family proteins such as Survivin and XIAP offers the anti-apoptotic therapeutic possibility by means of suppressing tumor growth in human cancer [48]. Recent studies have elucidated the complicated molecular mechanism of apoptosis, which has led to new targets and therapeutic strategies for treating diseases by overcoming the dysregulated apoptosis.

The viability of host cells in the protein-expression system is reduced by apoptosis in response to environ-

mental stimuli, such as viral infection, nutrient deprivation, growth factor withdrawal and oxygen limitation. With high density cultures of viable and productive cells, the inhibition of apoptosis results in an extended culture/production time period, which consequently leads to an improvement in the production capacity for valuable biotechnological products [18]. Nutrients, and survival other factors, supplemented to the cell culture medium rescues the apoptosis [49]. It has been shown that the productivity increases by extending the production time of host cells through the inhibition of apoptosis by *bcl-2* [50]. The overexpression of *bcl-2* resulted in enhanced humanized antibody production [51], and the constitutive expression of the antisense RNA of caspase 3 inhibited sodium butyrate-induced apoptosis [52]. Mammalian cells engineered to overexpress either *bcl-2* or *bcl-xL,* are resistant to virus infection [53] and various culture insults such as glucose and serum deprivation and toxic metabolite ammonia [54]. XIAP, the most potent caspase inhibitor encoded in the mammalian genome, and its deletion mutants exhibited the resistance to apoptosis in mammalian cell culture [55]. It has been reported that the viability of mouse myeloma NS0 cells was increased by the transfection with *Hsp70* [56] and *E1B-19K* [57], and this resulted in higher productivity. Some other anti-apoptotic genes have been also found to protect against apoptosis in a variety of cell line, and several peptide inhibitors of caspases have recently been reported to inhibit apoptosis in response to various apoptotic stimuli so far.

### Anti-apoptosis Engineering Using Proteins and Genes from Silkworm, *Bombyx mori*

Silkworm hemolymph, the most studied of all insect hemolymph, was used as a substitute for fetal bovine serum (FBS) [58-60]. It has been reported that, supplementing a medium with silkworm hemolymph increased the production of recombinant β-galactosidase up to 4.5-fold [61], and the longevity of host cells by inhibiting baculovirus-induced insect cell apoptosis [62]. The inhiition of apoptosis by silkworm hemolymph has been qualitatively observed using DNA ladder and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays [63], and quantitatively by flow cytometric analysis [64]. It has been found that silkworm hemolymph inhibits, not only baculovirus-induced apoptosis, but also apoptosis induced by various chemicals, such as actinomycin D, camptothecin and staurosporine [65]. It has been also shown that apoptosis-inhibition effect of silkworm hemolymph works in insect, mammalian and human cell systems [66]. This indicates that silkworm hemolymph contains a component that inhibits apoptosis.

To isolate the apoptosis-inhibiting component from silkworm hemolymph, it was fractionated by column chromatography, and each fraction tested for anti-apoptotic activity [67]. A database search, using the N-terminal amino acid sequence of the protein with the highest activity as a template, resulted in a 95% homology with one of the so-called '30 K proteins'. The 30 K proteins are a specific type of plasma protein called 'storage proteins'; they are a group of structurally related proteins, with molecular weights of approximately 30,000 Da [68]. Synthesized in the fat body of the feeding larvae, these are released into the hemolymph. During the larval-pupal transformation, they are transported from the hemolymph to the fat body cells, and stored as protein granules [69]. The 30 K protein group consists of 5 proteins, with common amino acid composition and immunological activity, as well as molecular weight characteristics. This group of proteins exhibit anti-apoptotic activity [67].

The cDNAs coding for each proteins, consisting of the 30 K protein, were constructed [70]. *30 Kc6* was used as a representative the 30 K protein cDNA in order to investigate the effect of its expression on apoptosis [71]. The 30 K protein was expressed in HEK293 and CHO K1 cells by transfection with the vector containing the *30 Kc6*. The HEK293 cell apoptosis, induced by staurosporine, was inhibited when the 30 K protein was transiently expressed. In the case of CHO K1 cell lines expressing the 30 K protein, an apoptosis-inhibition effect and a lower intracellular activity of caspase 3 were also found. This means that when the 30 K protein is expressed in the cells apoptosis is inhibited.

The 30 K protein, encoded by the *30 Kc6,* was expressed in *Escherichia coli,* purified, and added to a culture medium. Apoptosis was inhibited by supplementing the culture medium with this recombinant 30 K protein [71]. The recombinant 30 K protein inhibited both the virus- or chemical-induced mammalian and insect cell apoptosis. The apoptosis-inhibitory activity of recombinant the 30 K protein was comparable with that of the whole silkworm hemolymph. The recombinant 30 K protein produced in *E. coli* can be used like the authentic anti-apoptotic proteins from silkworm hemolymph.

The insect cell-baculovirus system has been used for the production of recombinant proteins [72,73]. This system offers several advantages, including a high expression, due to a strong polyhedrin promoter, the production of functionally and immunogenetically active recombinant proteins, as a result of proper post-transcriptional modifications, and the nonpathogenecity of the baculovirus to vertebrates and plants [74]. To maximize the production of recombinant proteins in this system, the host cell viability and longevity, after infection with baculovirus, are important in extending the productive time. To express the 30 K protein in insect cells, the recombinant baculovirus, producing the 30 K protein (AcNPV/30 K), was constructed and used to infect the insect cell. The viability of AcNPV/30 K-infected cells was as high as when supplementing with whole silkworm hemolymph in the early infection phase [71]. This means the expression of the 30 K protein is also effective in insect cell systems.

## CONCLUSION

Intensive research on apoptosis has identified various

**Table 1.** Anti-apoptotic effects of silkworm hemolymph and 30 K protein in various animal cell systems

| | Silkworm hemolymph | 30 K protein | |
|---|---|---|---|
| | Medium supplement | Intracellular expression | Medium supplement |
| Virus-induced apoptosis | Insect cells; Sf9 (Baculovirus) | Insect cells; Sf9 (Baculovirus) | Insect cells; Sf9 (Baculovirus) |
| | Human cells; HeLa (Vaccinia virus) | | |
| Drug-induced apoptosis | Insect cells; Sf9 (Actinomycin D, Camptothecin, and Staurosporine) | | Insect cells; Sf9 (Actinomycin D) |
| | Mammalian cells; CHO K1 (Staurosporine and Cisplatin) | Mammalian cells; CHO K1 (Staurosporine) | |
| | Human cells; HeLa and HEK293 (Stauarosporine) | Human cells; HEK293 (Staurosporine) | Human cells; HeLa (Staurosporine) |

anti-apoptotic proteins, such as the Bcl-2 family and IAPs (inhibitor of apoptosis proteins). The regulation of apoptosis, using anti-apoptotic proteins, has progressed the understanding of their inhibition mechanisms. This anti-apoptosis engineering can be used to treat the diseases related to apoptosis, and improve the productivity of animal cell cultures. Novel anti-apoptotic proteins have recently been isolated from silkworm hemolymph. They are 30 K protein group, consisting of 5 proteins, which have common amino acid composition and immunological activity, as well as molecular weight characteristics. The 30 K protein inhibits apoptosis induced by death stimuli in various systems. In this article we have described the effects of silkworm hemolymph and the 30 K protein originating from the silkworm, for various animal cell systems, and the anti-apoptotic effects of silkworm hemolymph and 30 K protein are summarized in Table 1. Anti-apoptosis engineering, using the 30 K protein and the corresponding gene, will provide a new anti-apoptosis strategy for therapeutic approaches and industrial applications of animal cell cultures. Although, further study is still required to elucidate the apoptosis inhibition mechanism associated with them.

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