Volume 59(Suppl.1):143-156, 2015
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

REVIEW

Genetics, molecular and cell biology of apoptotic cell death

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ABSTRACT Apoptotic cell death is an integral part of development and cell turnover in multicellular organisms. Since early 1970's, when apoptosis was defined on morphological basis, plethora of genes has been identified participating in initiation, execution and regulation of cell death. This article reviews these latest advances and describes our present understanding of the sequential events of apoptotic cell death, from the early steps of death receptor initiated and mitochondrial pathways to activation of caspases, and finally, the proper corpse clearance. It also discusses dysregulation of apoptosis, leading to various pathologies, such as cancer, autoimmune disease and neurodegenerative disorders. **Acta Biol Szeged 59(Suppl.1):143-156 (2015)**

KEY WORDS

apoptosis
caspases
death receptors
mitochondrial pathway
programmed cell death

Introduction

Multicellular organisms have evolved strictly regulated mechanisms for eliminating damaged, dangerous or surplus cells. This physiological form of cell death was originally described based on its characteristic cytological morphology and named apoptosis (Kerr et al. 1972), discriminating it from the accidental cell death, necrosis. As this process plays an essential role in normal embryonic development and adult tissue homeostasis and it is determined by genetic regulations, therefore it is also called programmed cell death.

The morphological changes of the apoptotic cells manifest with pseudopodia retraction, detachment from the substrate, reduction of cellular and nuclear volume (pyknosis), nuclear fragmentation (karyorrhexis), blebbing of the plasma membrane and package of the cell debris into apoptotic bodies (Kroemer et al. 2009), which are then engulfed by phagocytes *in vivo*. In contrast, necrotic cells undergo swelling of cytoplasmic organelles, including their nuclei, and the increase in their cell volume (oncosis) leads to rupture of the plasma membrane and loss of the intracellular material (Kroemer et al. 2009). Since the first description, it became clear that there are many "intermediate" forms of cell death between the classical apoptosis and necrosis, which can be more precisely distinguished based on molecular pathways. The new functional classification defines six main categories: extrinsic apoptosis, caspase-dependent intrinsic apoptosis, caspase-independent intrinsic apoptosis, regulated necrosis, autophagic cell death and mitotic catastrophe (Galluzzi et al. 2012).

In this review we will focus on apoptotic pathways, triggered either from outside of the cell by death ligands (extrinsic or death receptor pathway) or from inside the cell as a response to various stress signals (intrinsic or mitochondrial pathway) (Fig. 1). In both pathways, signalling converges on the activation of cysteine proteases, named caspases, which burst a proteolytic cascade, leading to concerted destruction and elimination of the dying cell.

Apoptosis induction by death receptors

The so-called "extrinsic apoptosis" is induced by extracellular death ligands which crosslink specific transmembrane death receptors. Death ligands belong to the of the Tumour Necrosis Factor (TNF) superfamily, which includes CD95 ligand/ Fas ligand (CD95L/FasL), tumour necrosis factor α (TNFα) and TNF-related apoptosis inducing ligand (TRAIL), expressed predominantly by immune cells (Guicciardi and Gores 2009). Accordingly, death receptors are members of the Tumour Necrosis Factor Receptor (TNFR) superfamily, transmitting decisive intracellular signals, leading to cell survival, activation, differentiation, inflammatory response or cell death (Guicciardi and Gores 2009). Only TNF receptors with death domains (DD) recruit the signalling elements of the death pathway, namely the CD95/Fas, TNF-R1, DR3, TRAIL-R1, TRAIL-R2, DR6, EDAR and NGFR (Aggarwal 2003). These receptors are present on the cell surface

Submitted March 8, 2015; Accepted June 5, 2015
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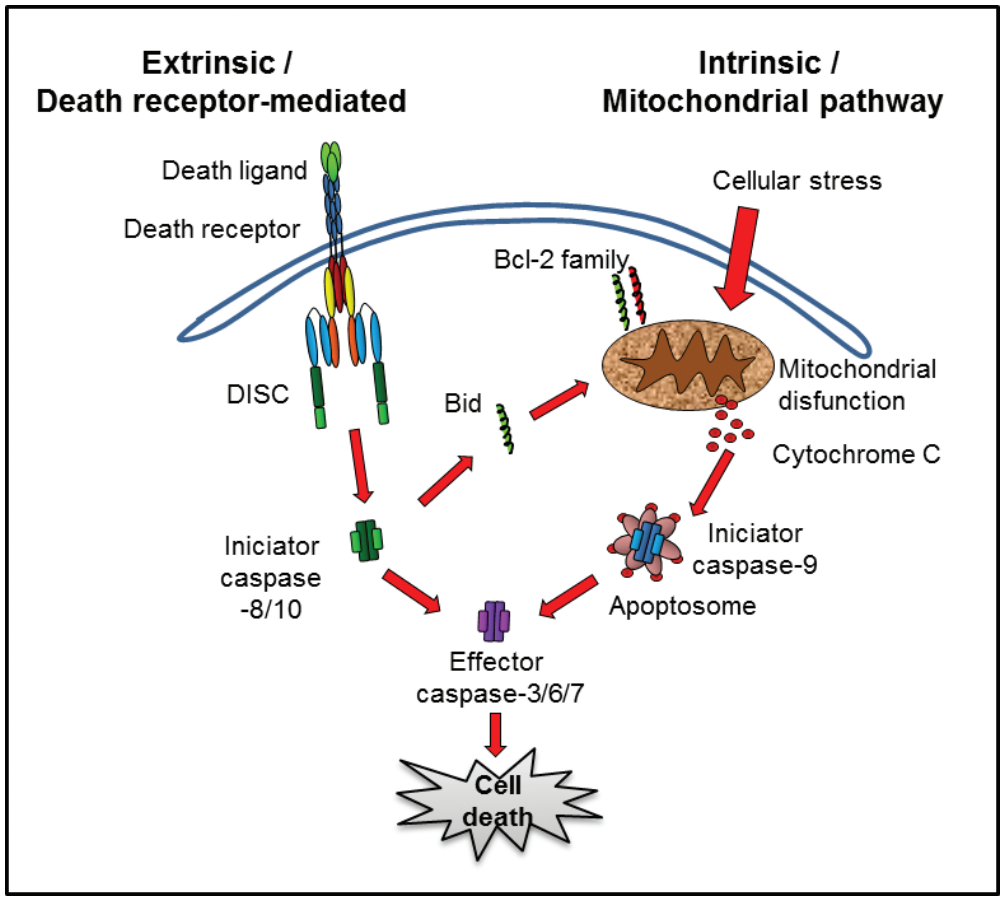

Figure 1. The extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway is triggered by death ligands, which bind to their specific death receptors in trimeric form. Ligation induces conformational change on the intracellular part, which then recruits adaptor proteins and initiator caspases, forming a large protein complex, named Death Inducing Signalling Complex (DISC). DISC is the platform for dimerization and activation of initiator procaspases-8 and -10. The intrinsic pathway is initiated by various cellular stresses, sensed by members of Bcl-2 family. Changes in the localization of Bcl-2 family proteins induce permeabilization of the mitochondrial outer membrane and release of cytochrome C. Cytochrome C facilitates assembly of apoptosome, where the initiator procaspase-9 is recruited and activated. Bid is cleaved by caspase-8 and, as a Bcl-2 family member, it promotes permeabilization of mitochondria, thereby providing a link between the extrinsic and intrinsic pathways and enhancing the apoptotic response. Initiator caspases trigger a proteolytic cascade of caspases, leading to activation of effector caspases, which are responsible for execution of cell death.

as trimers, through the interaction of the membrane-distal cysteine-rich domains of the extracellular part of the receptors, a region termed the preligand assembly domain (PLAD, Chan et al. 2000). However, binding of the ligand is required for stabilizing the preassembled receptor and triggering the downstream processes.

Signalling by CD95 ligand/ Fas ligand and CD95/ Fas interaction

Binding of CD95 ligand/ Fas ligand (CD95L/FasL) to CD95/ Fas leads to conformational change, which promotes assembly of a large protein complex, the Death Inducing Signaling Complex (DISC) (Fig. 1 and Fig. 2A) (Guicciardi and Gores 2009). In DISC, adaptor proteins, procaspases and their inhibitors are recruited based on homotypic molecular interactions between homologous domains. To the death domains (DD) present in the intracellular part of Fas, the adaptor protein FADD (Fas Associated Death Domain) is recruited by its homologous DD (Fig. 2A) (Chinnaiyan 1995). In addition, FADD contains another conserved domain, the Death Effector Domain (DED), which is found also in the large prodomain of procaspases-8 and -10 (Fig. 2A). Hence, DISC is a supramolecular complex where the initiator procaspases are recruited to, providing a platform for their dimerization and activation. However, inhibitors are recruited to this platform as well (Fig. 2A). The FLICE-like Inhibitory Protein (FLIP) has several isoforms that may regulate the dynamics of DISC

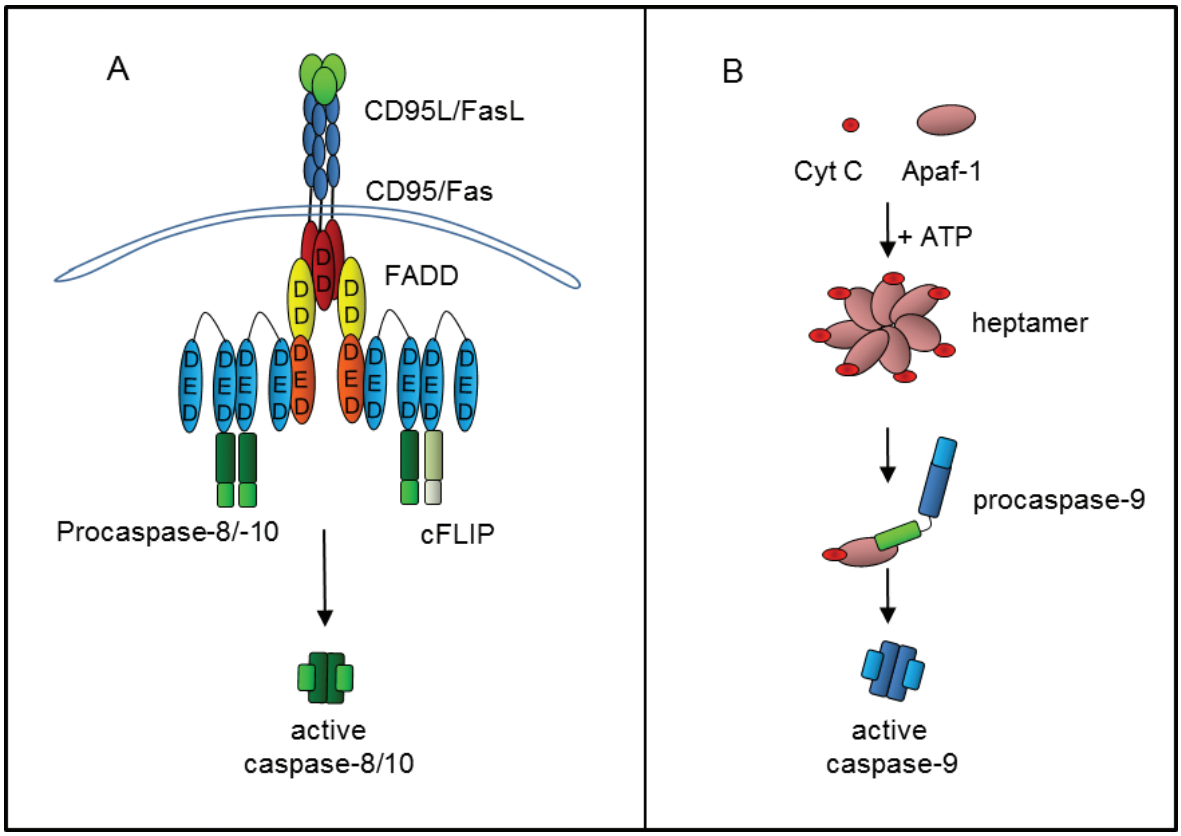

Figure 2. Mechanism of initiator caspase activation. A. Upon ligation by death ligands (i.e. CD95L/FasL), death domains (DD) in the intracellular tail of death receptors (i.e. CD95/Fas) recruit adaptor proteins with homologous DD, such as FADD (Fas Associated Death Domain). The other functional domains of these adaptors are the death effector domains (DED), which interact with DED prodomains of initiator procaspases -8 and -10. Recruitment of procaspases promotes their dimerization and autoproteolysis into large and small domains, thereby enhancing their activation. DED domains also recruit inhibitors like cFLIP (FLICE-like Inhibitory Protein), which hinders recruitment and dimerization of procaspases by sterical inhibition. B. Cytochrome C induces an opened conformation of Apaf-1 (Apoptotic protease-activating factor 1) and promotes its heptamerization in the presence of ATP, forming an apoptosome. The CARD domain of Apaf-1 recruits the CARD-domain containing initiator procaspase-9, and provokes its active conformation and cleavage.

composition and caspase activation (Safa 2012). cFLIP has similar structure as procaspase-8, therefore it is recruited to DISC by its DED domain, dimerizes with procaspase-8, but it has a mutant protease domain which inhibits the autocatalytic cleavage and activation of the protease. The outcome of the signalling pathway is largely dependent on the stoichiometry of the signalling elements present in DISC (Peter 2004). This basic structure is further complicated with additional signalling proteins, such as the receptor-interacting protein kinase 1 (RIP1) and the cellular inhibitor of apoptosis proteins (cIAPs), which can shift the cellular response from apoptotic signal to survival and inflammation by activating NF-κB pathway (Wang et al. 1998). RIP1 is a DD-containing serine/threonine kinase, which binds to all death receptors and DD-containing adaptors (Meylan and Tschopp 2005). RIP is polyubiquitinated and thereby activated by cIAPs, which are E3 ubiquitin ligases, leading to phosphorylation and degradation of NF-κB inhibitory protein IκBα, allowing NF-κB to translocate to the nucleus and initiate transcription of anti-apoptotic target genes, such as cFLIP, cIAP-1, cIAP-2, TRAF1 and TRAF2 (Tumor Necrosis Factor Receptor Associated Factor 1 and 2) (Wang et al. 1998), hence generating a positive feedback loop of pro-survival signalling.

Dichotomy of the TNF/TNFR signalling

In contrast to Fas, TNF-R1 is primarily involved in inflammatory immune response and not in apoptosis. Ligation of TNF-R1 triggers assembly of two subsequent signalling complexes (Guicciardi and Gores 2009). The first complex (complex I) consists of adaptor proteins TRADD (TNFR Associated Death Domain), RIP1, TRAF2 and/or TRAF5 adaptor proteins and cIAP1/2 ubiquitin ligases, and results in activation of downstream NF-κB, JNK and p38 pathways.

The proteins upregulated by these transcription factors work against death signalling, and provide a regulatory mechanism to control cell death. Only when TNF-R1 is internalized after ligation, the pro-survival complex I dissociates, allowing FADD and procaspase-8 and -10 to form a cytosolic TNF-R1-associated DISC (complex II) and initiate the downstream apoptotic processes (Guicciardi and Gores 2009). Endocytosis of TNF-R1 is decisive for assembly of complex II, and it is regulated by a cytoplasmic region named TNF-R1 internalization domain (TRID) (Schneider-Brachert et al. 2004). The temporal and spatial distinction of complex I from complex II provides a checkpoint to control cell death, as the activation of initiator caspases may be diminished by the complex I-driven, NF-κB-dependent cFLIP expression. TNF-induced apoptosis is enhanced in the presence of protein synthesis inhibitors (cycloheximide), indicating the importance of the newly expressed anti-apoptotic proteins, and it is also promoted by administration of cIAP inhibitors *in vitro*. Hence, the outcome of TNF-R1 ligation largely depends on the status of the cells, *i.e.* it can engage either the pro-survival NF-κB or the pro-death caspase signalling pathway.

Outcomes of TRAIL signalling

TRAIL/Apo2L triggers apoptosis by binding to one of its two cognate death receptors, TRAIL-R1 and TRAIL-R2 (Pan et al. 1997), expressed in most human tissues, including spleen, thymus, liver, peripheral blood leukocytes, activated T cells, small intestine, and some tumour cell lines. In addition, it has two decoy receptors, DcR1 and DcR, which lack functional intracellular death domains (Sheridan et al. 1997). Binding of TRAIL to decoy receptors fails to trigger apoptosis, moreover, it prevents TRAIL-induced apoptosis through TRAIL-R1 and TRAIL-R2 by inhibiting the recruitment and activation of death signalling elements to DISC, and by shifting the cell response toward NF-κB activation (Degli-Esposti et al. 1997). Apparently, most healthy cells are protected from TRAIL-triggered apoptosis by expressing decoy receptors, and TRAIL plays non-apoptotic functions in immunoregulation. Expression of both TRAIL and its receptor on cells of the innate and adaptive immune systems is dependent on the stimulation status and it is modulated by viral and bacterial infections. Results with TRAIL$^{-/-}$ and TRAIL-R$^{-/-}$ mice suggest that this ligand/receptor system has been primarily developed to fight infections and to control immune responses (Falschlehner et al. 2009).

Regulation of mitochondrial pathway by members of Bcl-2 family

The activation of the intrinsic death pathway is triggered by various stress signals that modify the cellular level and localization of Bcl-2 protein family members, controllers of the integrity of mitochondrial membrane. The imbalance in the anti-apoptotic and pro-apoptotic members of Bcl-2 protein family and the integration of pro-apoptotic ones into the outer membrane of mitochondria lead to release of cytochrome C (cyt C) from the mitochondrial intermembrane space, required for the subsequent formation of the apoptosome, a catalytic multiprotein platform for activation of caspase-9 (Fig. 1 and Fig. 2B) (Bender and Martinou 2013). Today, three functionally and structurally distinct subgroups of the Bcl-2 protein family are defined: 1. cell death initiating BH3-only proteins; 2. the anti-apoptotic members such as the Bcl-2 itself; 3. the pro-apoptotic executioner proteins Bax and Bak (Bender and Martinou 2013). All family members share common Bcl-2 homology domains (BH), however, the anti-apoptotic proteins and the pro-apoptotic Bax/Bak have four BH domains, while the BH3-only proteins have only one (Bender and Martinou 2013).

The anti-apoptotic Bcl-2 proteins

The Bcl-2 (B-cell lymphoma 2) anti-apoptotic protein was identified in patients with pre-B-cell leukaemia and follicular lymphoma carrying a chromosome translocation, which resulted in overexpression of an oncogenic protein (see below, Tsujimoto et al. 1985). Shortly, it turned out that Bcl-2 protected hematopoietic cells from cell death (Vaux et al. 1988) and that it was a functional homologue of CED-9, which prevented developmental cell death in *C. elegans* (Hengartner and Horwitz 1994). To date, six human pro-survival family members have been identified: Bcl-2, Bcl-XL, Bcl-W, MCL-1, A1 and Bcl-B (Czabotar et al. 2014). These proteins have four BH domains and a transmembrane domain (TM) located near the carboxyl terminus which anchors them to intracellular membranes of mitochondria and other organelles. It is suggested that the anti-apoptotic proteins bind and sequester the pro-apoptotic proteins, including the activator BH3-only proteins and Bax and Bak, to prevent apoptosis (Czabotar et al. 2014).

The role of BH3-only proteins

The BH3-only proteins are the sensors of stress signals and the molecular switch to apoptotic pathway (Fig. 3), as they interact with the multiregion pro-apoptotic and anti-apoptotic Bcl-2 family members, regulating their functions. There are at least 10 different BH3-only proteins in the vertebrate genome (Aouacheria et al. 2005) with different subcellular localization and cell type-specific expression, induced by many different types of cell stress. The specificity and affinity of the BH3-only proteins for binding with their partners are determined by small differences in the amino acid sequence in the BH3

region. Only the so called "direct activators" – such as Bid, Bim, Puma – can directly bind to executioners, Bax and Bak. Other BH3 proteins (such as Bad, Bmf, Bik, Hrk, Noxa) are designated as "sensitizers", as they have been shown to bind the anti-apoptotic Bcl-2 proteins, thereby blocking them from binding to the activator BH3 proteins and executioner proteins (Häcker and Weber 2007). As BH3-only proteins play crucial role in initiating mitochondrial pathway of apoptosis, their activation is under strict regulation. BH3-only proteins are regulated by their cellular localization, posttranslational regulation - such as phosphorylation, myristoylation, ubiquitination, proteolysis - or at transcriptional level. For example, Bim and Bmf are anchored to cytoskeleton in healthy cells (Puthalakath et al. 1999); Bad is sequestered by 14-3-3 when phosphorylated upon signalling from growth factors (Zha et al. 1996); Puma and Noxa are targets of p53 transcription factor (sensor of DNA damage – see below) (Nakano et al. 2001; Oda et al. 2000); and Bid is cleaved by caspase-8 (Fig. 1) providing an amplification loop for death ligand-induced pathway (Li et al. 1998).

According to the "embedded together" model, the sensitizer BH3-only proteins bind the anti-apoptotic Bcl-2 proteins and liberate the activator BH3-only proteins and the executioner pro-apoptotic proteins, Bax and Bak (Fig. 3), allowing the activation and homo-oligomerization of the latter (Bender and Martinou 2013).

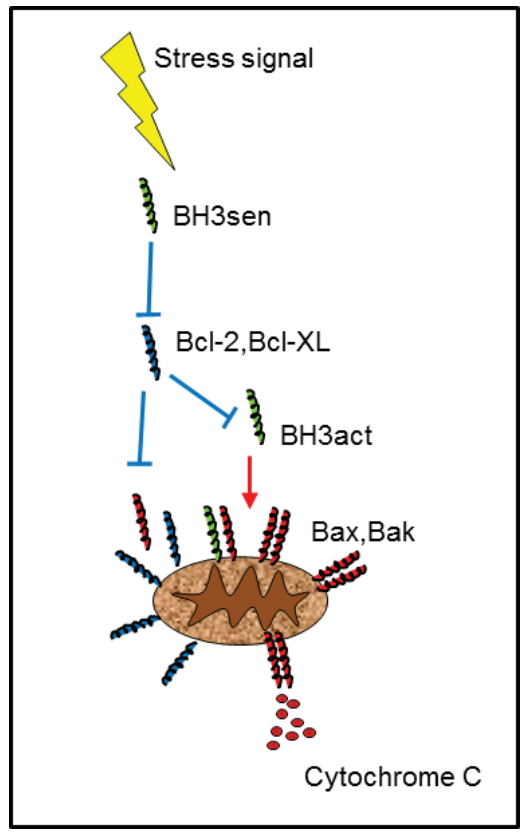

Figure 3. Members of Bcl-2 protein family regulate mitochondrial pathway. Various stress signals trigger changes in localization and expression of BH3-only proteins, which are the initiators of the mitochondrial apoptotic pathway. Sensitizer BH3-only proteins (BH3sen) bind to anti-apoptotic Bcl-2 proteins (i.e. Bcl-2 and Bcl-XL) and inhibit their guardian function. This releases the activator BH3-only proteins (BH3act) and the pro-apoptotic Bax and Bak from the inhibition of Bcl-2. BH3act proteins directly bind Bax and Bak and trigger their embedding to the outer membrane of mitochondria. Oligomers of Bax and Bak develop pores suitable for release of cytochrome C from intermembrane space.

The pro-apoptotic function of Bax and Bak

Bax and Bak are the multiregional Bcl-2 family members that form pores on the outer mitochondrial membrane, directly involved in releasing the pro-apoptotic factors from mitochondria (Bender and Martinou 2013). Both Bax and Bak are needed for maintaining normal development and tissue homeostasis, indicating that these proteins have overlapping functions (Lindsten et al. 2000). Bak resides in the outer mitochondrial membrane, but its oligomerization is inhibited by Bcl-XL in healthy cells (Griffiths et al. 1999). In contrast, Bax is predominantly a soluble protein in healthy cells cycling regularly to mitochondria, while its transmembrane domain is attached to its hydrophobic grove and unable to dock in membranes (Wolter et al. 1997). Upon apoptotic trigger, binding of activator BH3-only proteins induces conformational change of Bax and it redistributes it to mitochondria (Griffiths et al. 1999; Wolter et al. 1997), were it contributes to forming pores. The exact mechanism how Bax and Bak permeabilize the mitochondrial outer membrane is still under debate (Czabotar et al. 2014; Shamas-Din et al. 2013). During apoptosis, Bax and Bak change their structure and form homo-oligomers that can permeabilize the mitochondrial outer membrane.

Recently, a second hydrophobic pocket of Bax was identified, termed the "rear pocket", located on the opposite side from the canonical "front" BH3-binding pocket of Bax. It is suggested, that this pocket is masked when Bax is inactive and soluble in the cytoplasm, and it becomes exposed when Bax binds an activator BH3-only protein, triggering multiple conformational changes (Gavathiotis et al. 2010), leading to formation of pores, termed mitochondrial apoptosis-induced channel (MAC) (Pavlov et al. 2001). Current results show that Bax and Bak generate both proteinaceous and lipidic pores, large enough for releasing intermembrane space proteins, such as cytochrome C, OMI/HTRA2 and SMAC/DIABLO (Shamas-Din et al. 2013) (Fig. 3).

Activation of caspases

Both the death receptor and mitochondrial pathways trigger activation of the caspase cascade (Fig. 1, Fig. 2A and B). Caspases are a family of endopeptidases, having catalytic cysteine residues in their active site and cleave after certain aspartic acids in the substrate bearing a consensus sequence (Riedl and Shi 2004). Fourteen mammalian caspases have been identified so far; however, they have different functions in regulating homeostasis, such as in differentiation, inflammation or cell death. Apoptotic caspases are classified by their mechanism of action to group of initiators (caspases-2, -8, -10 and -9) and effectors (caspases-3, -6 and -7). Caspases-1, -4, -5 and -12 in humans and caspase-11 in mice have roles in inflammatory responses and discussed elsewhere (Riedl and Shi 2004).

The caspase cascade

As caspases are the masters of cell degradation, their activities are regulated on multiple levels. Caspases are produced in inactive, zymogen form as procaspases, and require dimerization and cleavage for activation (Riedl and Shi 2004) (Fig. 2). Dimerization of initiator caspases takes place on large molecular complexes assembled upon death receptor ligation or cyt C release from mitochondria. Procaspase-8 is recruited to DISC by its DED prodomain, and its dimerization facilitates autocatalytic cleavage into large and small subunits resulting in stabilization of the dimer (Fig. 2A). This process has been described as an "induced proximity model" (Muzio et al. 1998). On the mitochondrial pathway, release and accumulation of cyt C in cytoplasm trigger conformational change of Apaf-1 (Apoptotic protease-activating factor 1) and, in the presence of cofactor dATP, they assemble to a large molecular complex, named apoptosome (Fig. 2B). Apaf-1 has three distinct domains: an N-terminal CARD for oligomerization; an expanded nucleotide-binding domain for dATP binding; and WD40 repeats for interaction with cyt C (Zou et al. 1997). Apoptosome has a wheel-like crystal structure, composed from seven units, with CARD domains in the middle, and WD40 on distal arms (Acehan et al. 2002). Homotypic interaction of CARD domains is also responsible for recruitment and activation of procaspase-9, which possesses a homologous CARD prodomain. In a recent study conducted with purified Apaf-1 and procaspase-9 CARD domains, an 11-mer complex was identified composed of seven molecules of Apaf-1 and four molecules of caspase-9, suggesting additional interfaces between these proteins (Hu et al. 2014). Surprisingly, procaspase-9 bound to the apoptosome exhibits high catalytic activity even in the absence of the activation cleavage, which indicates allosteric regulation of its catalytic activity (Stennicke et al. 1999) and supports the induced conformation model. Once activated, the initiator caspases-8 and -9 can initiate a caspase cascade involving the downstream executioner caspases-3, -6 and -7 (Fig. 1). The effector caspases form dimers in their zymogen form, but require specific intrachain cleavage, leading to a conformational change in a critical loop in one caspase monomer to stabilize the active site loops in the adjacent monomer (Fig. 4) (Riedl et al. 2001).

Regulation of caspase activation

In addition to keeping caspases in zymogenic form until the apoptotic stimuli, various other mechanisms have been described for regulation of caspase activity. As discussed above, inhibitory protein cFLIP and its isoforms block recruitment and dimerization of procaspase-8 in DISC. Activation of the pro-survival NF-κB signalling pathway enhances production of these inhibitory proteins and promotes expression of IAPs, too. IAPs are a conserved family of proteins, identified by bearing baculovirus IAP repeat domain (BIR) (Gyrd-Hansen and Meier 2010), which has a zinc-binding fold of approximately 70 amino acid residues that mediates protein–protein interactions (Hinds et al. 1999). The amino-terminal of mammalian IAPs, such as XIAP, cIAP1 and cIAP2 proteins contain three BIR domains, further classified to type I BIR and two type II BIR domains (Gyrd-Hansen and Meier 2010). Type II BIR domains have a deep peptide-binding groove for interaction with proteins carrying an IAP-binding motif (IBM), and have differential binding preferences for specific IBM-carrying proteins. The linker region preceding BIR2 in XIAP binds to the IBMs of caspase-3 and caspase-7, in contrast, BIR3 binds to caspase-9, and their mechanism of inhibition is dissimilar (Gyrd-Hansen and Meier 2010). The BIR2 domain of XIAP binds to the neo-amino-terminus of effector caspases exposed following cleavage-mediated activation of caspase-3 and caspase-7 (Scott et al. 2005), while BIR3 binds to the homodimerization surface of caspase-9, interfering with dimerization-induced activation of this initiator caspase (Shiozaki et al. 2003). Mammalian IAPs also contain a carboxy-terminal RING domain indicating E3 ubiquitin (Ub) ligase function and a Ub-associated (UBA) domain, and interfere with survival signalling pathways, such as NF-κB (see above). The caspase inhibition by IAPs can be overcome by their antagonists carrying IBM domains, such as the mitochondrial apoptotic factors, Smac/DIABLO and OMI/HTRA2, as they bind to the BIR domains and block their access to caspases (Vaux and Silke 2003), therefore enabling the caspases to execute their effective function.

Targets of caspases

Caspases' substrate specificity is defined by 4-5 consensus amino acid sequence, with aspartic acid at the P1 primary

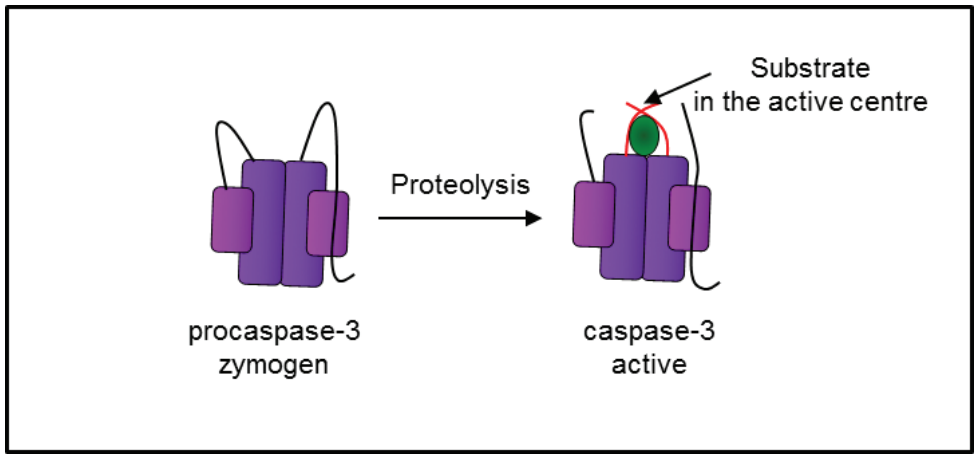

Figure 4. Mechanism of effector caspase activation. Effector caspases form dimers in their zymogen form, but they are not active unless cleaved by initiator caspases. Cut in the loop between the large and small domains induces conformational change in the active site of the dimerization partner, stabilizing it and enhancing its enzymatic activity.

specificity position. This sequence is common for group of caspases, for example DEXD for effector caspases-3 and-7, and I/LEXD for initiator caspases-8 and -9 (D - Asp, E - Glu, I - Ile, L - Leu, X - any AA) (Poreba et al. 2013). This short sequence is present in several hundreds of proteins, putative caspase substrates, reported from modern, sensitive and high-throughput proteomics studies (Crawford and Wells 2011). However, caspase cleavages are not all destructive, but as limited proteolysis they have different effects on different substrates: they can cause loss, gain or change in target protein's function or change in its localization (Crawford and Wells 2011). Examples for gain-of-function cleavage are the activation of caspases themselves, but also the truncation of Bid, a BH3-only protein or the degradation of the inhibitor of the caspase-activated DNAse (CAD). The latter liberates CAD to catalyse fragmentation of nuclear DNA, which is considered as one of the hallmarks of apoptosis (Nagata 2000). Loss-of-function cleavages degrade structural proteins (such as lamin, a nuclear structure protein), impair enzymes involved in energy-consuming synthesis processes that are no longer needed in a dying cell, and destroy activity of transcription factors participating in growth and differentiation programs (Crawford and Wells 2011). Cleavage by caspases can alter the subcellular localization of proteins by removing signal peptides. For example, Abl is a tyrosine kinase involved in cell survival signaling pathway in the cytoplasm, however, in response to DNA damage it concentrates in the nucleus and phosphorylates p53 and p73 transcription factors, which enhance expression of proapoptotic factors (see below). The shift of Abl toward nuclear localization is facilitated by the cut of its nuclear export signal (NES) by caspases-3, -7, thereby enhancing the apoptotic signalling (Barila et al. 2003).

Clearance of apoptotic cells

Apoptosis does not end with the structural degradation of the cells and fragmentation to small apoptotic bodies. Clearing of the apoptotic debris is at least as important as the triggering and signalling phases, since it protects from emerging of inflammatory and autoimmune responses (Hochreiter-Hufford and Ravichandran 2013). Without clearing, apoptotic bodies undergo secondary necrosis, i.e. rupture of the membrane and leakage of intracellular material, as it can be followed in the cell-cultures *in vitro*. In contrast, apoptotic bodies are rarely detected events in histological sections even in tissues with high cellular turnover, as they are rapidly engulfed by phagocytes *in vivo*. This can be performed by any cell type, which is capable of engulfment, including "professional" phagocytes, such as macrophages and immature dendritic cells, and the neighboring "nonprofessional" phagocytes in all tissues (Hochreiter-Hufford and Ravichandran 2013). Clearance has a regulated choreography: first, apoptotic cells release so called "find me" signals, which are chemotactic factors for phagocytes. Dying cells also express "eat-me" signals, which provide contact with and recognition by the phagocytes, triggering signals for the engulfment process. Finally, endocytosis of apoptotic bodies upregulates production of anti-inflammatory cytokines, keeping the clearance events "immunologically silent" (Hochreiter-Hufford and Ravichandran 2013) (Fig 5).

Recruitment of phagocytes

The "find-me" signals establish a chemotactic gradient

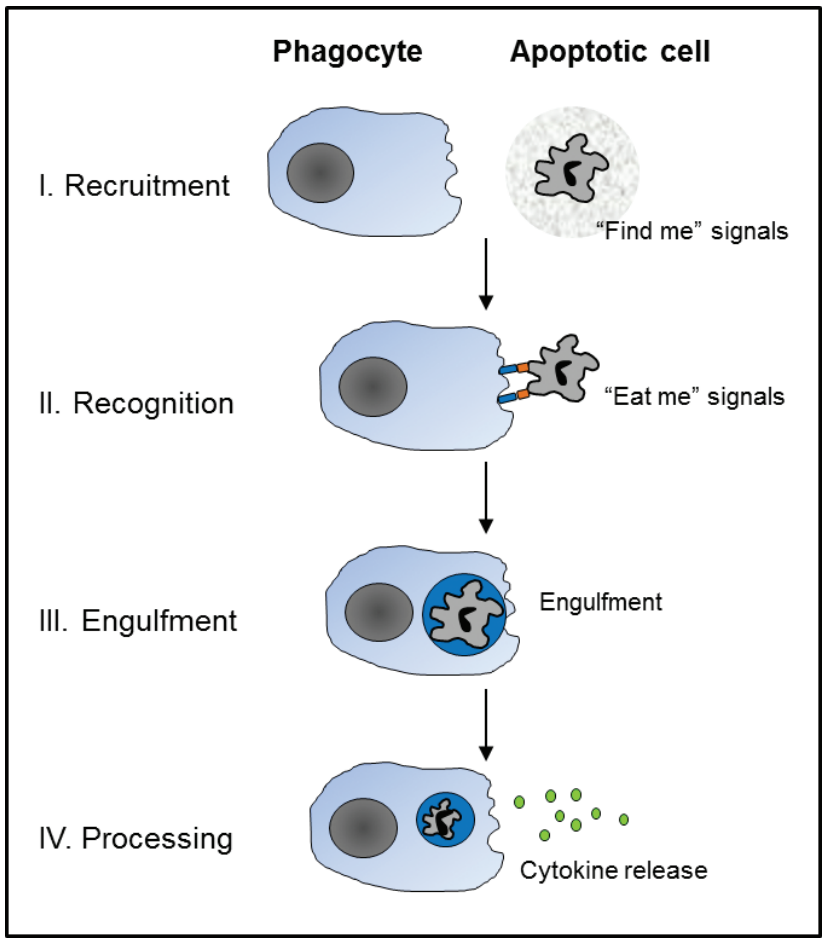

Figure 5. Phases of clearing of apoptotic cells. I. Dying cells release "find me" signals, which provide chemotactic gradient for recruitment of phagocytes. II. Changes in the surface pattern of apoptotic cells offer "eat me" signals, recognized by scavenger receptors of phagocytes. III. Activation signals triggered from scavenger receptors lead to cytoskeletal rearrangements and engulfment of apoptotic corpse. IV. Processing of endocytosed apoptotic debris promotes release of anti-inflammatory cytokines.

stimulating migration of phagocytes to the apoptotic cell. The most intensively examined "find-me" factors are the lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), fractalkine (CXC3CL1 chemokine) and the nucleotides ATP and UTP, however, their exact action is yet under debate (Hochreiter-Hufford and Ravichandran 2013). LPC was the first "find-me" signal described. It is a product of the caspase-3-dependent activation of phospholipase A2, which converts the membrane phosphatidylcholine to LPC (Lauber et al. 2003). However, the concentration of LPC reported to be required for macrophage chemotaxis appears to be quite high, making LPC an unlikely candidate for a chemotactic mediator. It also acts as an "eat-me" signal, being a surface-bound target for IgM. The other lipid mediator, S1P is produced by sphingosine kinase 1 (SphK1) (Gude et al. 2008), and stimulates chemotaxis, although it also seems to have additional function by enhancing the immunosuppressive factors, IL-10 and PGE2 by macrophages. Fractalkine is a membrane-associated protein released by B-lymphocytes and neurons, and it is sensed by the macrophage chemokine receptor, CX3CR1, hence it is suggested to be a chemotactic factor, however, its production is not uniform in every cell type. (Truman et al. 2008). Recently, nucleotides ATP and UTP have been proposed as a new class of "find me" signals. These are released is small amounts *via* the pannexin channels, opened as a result of cleavage by caspases, and they establish a short-range chemotactic gradient for tissue resident macrophages that express the nucleotide-sensitive P2Y2 receptor (Elliott et al. 2009).

Recognition of apoptotic cells and provoking engulfment

The molecular composition of the plasma membrane of an

apoptotic cell differs from healthy cells in displaying "eat me" signals, which are detected by phagocytes either directly with their scavenger receptors or with help of bridging molecules. In addition, living cells express "don't eat me" signals on their surface, like CD47 (also known as integrin-associated protein) and CD31, which inhibit engulfment by phagocytes (Oldenborg et al. 2000; Brown et al. 2002). In case of apoptotic cells, changes in glycosylation of surface proteins and in surface charge and expression of oxidized low-density lipoprotein (LDL)-like moiety provokes recognition by phagocytes directly or through binding of serum proteins such as thrombospondin and complement C1q (Ravichandran and Lorenz 2007). The most widely known "eat me" signal is the phospholipid phosphatidylserine (PtdSer) (Fadok et al. 1992), which is immersed in the inner leaflet of the lipid bilayer in viable cells, but it rapidly translocates to the outer leaflet in apoptotic cells by a caspase-dependent mechanism. Certain intracellular proteins such as calreticulin and annexin I, are also exposed and function as bridging molecules by binding to and enhancing the recognition of PtdSer (Arur et al. 2003; Gardai et al. 2005). Receptors expressed on the surface of phagocytic cells that recognize "eat me" signals include lectins that bind altered sugars on apoptotic cells, CD36 (in conjunction with integrins $\alpha v\beta 3$ and $\alpha v\beta 5$) that binds thrombospondin, LRP1/CD91 (in conjunction with calreticulin) that binds complement C1q, and the scavenger receptors that bind oxidized LDL (Hochreiter-Hufford and Ravichandran 2013). In addition, secreted proteins such as MFG-E8, growth-arrest-specific 6 (Gas6), and protein S have also been shown to bind PtdSer, and as bridging molecules they promote engulfment *via* their cognate receptors such as the Tyro-3-Axl-Mer family of receptors (TAM receptors) on phagocytes (Nakano et al. 1997).

Upon recognition of the apoptotic cell, the phagocyte undergoes cytoskeletal rearrangements necessary for corpse engulfment. Most phagocytic receptors are connected with the CrkII–Dock180–ELMO signalling pathway, having evolutionary conserved function in clearance. Dock180 is a specific guanine-nucleotide- exchange factor (GEF), responsible for activation of Rho family GTPases, such as Rac, which in turn promote actin polymerization and cytoskeletal rearrangement. Recognition and engulfment of apoptotic cells trigger secretion of anti-inflammatory cytokines (TGF-β and IL-10) from the phagocyte, which induce differentiation of regulatory T-cells and T helper 2 cells, thereby dampening or resolving inflammation (Green et al. 2009). In addition, degradation and processing of apoptotic cell material by professional phagocytes, such as dendritic cells, establish and maintain tolerance by modulating self-antigen presentation. Defects at any points of clearance (lack of phagocytic receptors, improper degradation after engulfment) may lead to development of wide variety of human pathologies, for example autoimmune diseases (systemic lupus erythematosus and rheumatoid arthritis), pulmonary diseases (chronic obstructive pulmonary disease and asthma), cardiovascular diseases (atherosclerosis), neurological diseases (Alzheimer's disease) and cancer (Elliott and Ravichandran 2010).

Table 1. Defective apoptosis leads to pathological disorders.

Suppressed apoptosis	Excessive apoptosis
Cancer:	*Neurodegenerative diseases:*
Lymphomas	Alzheimer's disease
Gastric	Parkinson's disease
Colorectal	Huntington's disease
Lung	Amyotrophic lateral sclerosis
Neuroblastoma	Neurodegeneration after stroke
Autoimmune disorders:	*Cardiovascular diseases:*
Systemic lupus erythematosus	Myocardial infarction
Myasthenia gravis	
Other disorders	*Other disorders*
Frequent viral infections	Inflammation
	Sepsis
	AIDS
	Type I diabetes

Malfunctioning of apoptosis

As apoptosis is a basic biological process in the maintenance of tissue homeostasis, alterations in apoptotic pathway can lead to various diseases. Both the reduced and increased apoptotic processes can be involved in the pathomechanism of cancer, several types of autoimmune diseases, neurodegenerative diseases and cardiovascular diseases (Table 1).

Defective apoptosis in cancer

Failure of apoptosis allows survival of transformed cells, which are prone to undergo further genetic damage. Suppression of apoptosis plays a central role in tumour progression and it may be responsible for resistance to cancer therapy as well (Lowe and Lin 2001). Cancer cells can decrease apoptosis *via* mutations in key regulatory genes, downregulation of expression of proapoptotic molecules or overexpression of apoptosis inhibitors as well. Surprisingly, the frequency of somatic mutations in caspase genes is relatively low in tumours. In genes of initiator caspases, *i.e.* caspase-8 and -10 the mutation rate is around 10% in various cancer types, however, it is only 2% in genes of effector caspases, like caspase-3, -6, and -7, represented only in a few tumour types (McIlwain et al. 2013). Genetic studies have shown that inactivation of individual caspases is usually not sufficient to block the caspase cascade. Instead, malignant cells more frequently inactivate the upstream mediators of caspase activation.

In contrary to caspase genes, the master regulator of apoptosis, TP53 tumour-suppressor gene is mutated in more than 50% of all tumour types (Wallace-Brodeur and Lowe 1999). These somatic mutations arise spontaneously or as a consequence of DNA damage in tumour cells. Mutations are usually missense, single base mutations in DNA-binding domain of TP53, which lead to loss of function of p53 protein (Whibley et al. 2009). In normal cells, p53 activates the intrinsic apoptotic pathway through transactivation or transcription-independent mechanism. In nucleus, p53 induce gene expression of several members of BH3-only protein family (Puma, Noxa and Hrk) as a transcription factor, while at mitochondria it interacts directly with members of the Bcl-2 family that leads to cytochrome C release and caspase-9 activation (Amaral et al. 2010). p53 also activates the extrinsic apoptotic pathway through the induction of genes encoding Fas, DR5 and Bid, the latter linking the extrinsic and intrinsic apoptotic pathways. Furthermore, p53 is involved in the activation of the apoptosome *via* induction of Apaf-1 expression (Amaral et al. 2010). The central role of p53 in tumourigenesis is indicated by germline TP53 mutations. Individuals with Li-Fraumeni syndrome harbouring germline TP53 mutations have an increased risk of developing various tumour types like sarcomas, breast cancers or brain tumours at an early age of onset. Similar to somatic mutations, germline Li-Fraumeni mutations are most often missense base substitutions in DNA-binding domain of TP53 (Malkin 1993).

While mutations in p53 lead to loss of normal functions in apoptosis regulation, the another significant regulator, Bcl-2 displays a gain of function phenotype. Bcl-2 is upregulated in a variety of tumour types resulting in imbalance between the anti-apoptotic Bcl-2 and pro-apoptotic Bax, subsequently leading to decreased intrinsic apoptotic pathway in cells. In most cases of B-cell lymphomas, the elevated level of Bcl-2 is a result of translocation between chromosome 14q32 region and chromosome 18q21 region, bringing the BCL-2 gene under the control of the immunoglobulin heavy-chain enhancer (Tsujimoto et al. 1985), hence giving eternal life for B-cells. Additional mechanisms for increased Bcl-2 expression occur in about 50% of all human cancers. Among these are the loss of endogenous microRNAs (miRs) that normally repress BCL-2 gene expression and altered epigenetic regulation due to gene hypomethylation (Yip and Reed 2008). In addition to involvement in tumourigenesis, decreased apoptosis may be responsible for drug resistance in cancer chemotherapy as well. Currently several compounds regulating apoptosis enter clinical trials to evaluate their efficacy in cancer treatment (Fischer et al. 2005).

TRAIL-receptor has been suggested to play role as a metastasis suppressor in multiple tissues based on the *in vivo* studies with TRAILR-deficient mice (Grosse-Wilde et al. 2008), which gives hope for TRAIL-based therapy in human cancer. However, the translation of TRAIL into the clinic turned to be aggravated by its short half-life, lacking of adequate delivery methods, and presence of TRAIL-resistant cancer cell populations (Stuckey and Shah 2013). Use of TRAIL-receptor targeting monoclonal antibodies has been successful in preclinical studies and entered clinical trials. The phase I trials of soluble recombinant TRAIL or TRAIL-R monoclonal antibodies have been undertaken on patients with advanced solid tumours, and these compounds were largely well tolerated, nevertheless, their anticancer response was poor, showing no remission. Currently, combination therapies are in ongoing trials, and their relative success remains to be evaluated (Stuckey and Shah 2013).

Problems with apoptosis in the nervous system

Programmed cell death is part of the normal nervous system development, but excessive apoptosis in adults plays role in the pathomechanism of several neurodegenerative diseases. Contrary to most of somatic cells, neurons do not replicate and they live for a long time, therefore, elevated level of apoptosis in certain neurons results in diminished cell number in a particular region of adult brain. For example, progressive loss of motoneurons manifests as Amyotrophic Lateral Sclerosis (ALS), characterized by muscle atrophy and paralysis; apoptosis of cortical and hippocampal neurons is responsible for the symptoms of Alzheimer's disease (AD) which is an irreversible, progressive dementia; Huntington's chorea (HD) involves death of neurons in the striatum that control body movements, resulting in abnormal involuntary movement; specific loss of dopaminerg midbrain neurons underlies Parkinson's disease (PD) characterized by lesions in the substantia nigra (Hee and Keun 2013). Elevated caspase activity is a common feature of neurodegenerative disorders. Caspase-1, -3 and -9 activities are higher in ALS patients than in controls (Hee and Keun 2013) and apoptosis in the motor neurons may contribute to the disease, although the molecular pathomechanism of ALS is not clear. The main feature in AD is amyloid β-peptide (APP) aggregation in extracellular deposits, causing neurotoxic plaques. Here, caspase-3 is indicated as the main caspase involved in mutant amyloid precursor protein cleavage (Gervais et al. 1999), and the resulting APP is considered to induce apoptosis by causing oxidative stress in neurons and glia. Moreover, the N-terminal APP fragment activates the extrinsic apoptotic pathway which leads to caspase-6 dependent axonal degeneration (Nikolaev et al. 2009). The exact mechanism of cell death in HD is unclear yet, but it is possible that peptide fragments of mutant huntingtin protein generated by caspase-6 cause the neuropathological symptoms (Graham et al. 2010). In PD animal models, involvement of caspase-1 and -3 in cell death has been proved, in addition, PTEN-induced kinase-1 (PINK-1) mutation results in elevated levels of capase-3 and -9 activation (Wood-Kaczmar et al. 2008). Better understand-

ing of the molecular mechanisms of neuronal apoptosis may promote introduction of anti-apoptotic drugs in therapy of neurodegenerative disorders in future.

Conclusive remarks

Our understanding of the regulation of apoptosis and its role in tissue dynamics of multicellular organisms has greatly expanded in recent years. Identification of protein families involved in execution and regulation of apoptosis boosted the studies defining their exact place and role in apoptotic signalling machinery. However, many questions regarding the structure of these proteins after conformational change, oligomerization and membrane anchorage, are still open. Uncovering the delicate interrelationship between the players of apoptosis gives tools to fight diseases with abnormal cell destruction or excessive cell survival.

Acknowledgements

This work was supported by the TÁMOP-4.1.1.C-13/1/KONV-2014-0001 program entitled "Practice-oriented, student-friendly modernization of the biomedical education for strengthening the international competitiveness of the rural Hungarian universities and by TÁMOP-4.2.2.A-11/1/KONV-2012-0035 Investigation of the interactions of environmental and genetic factors in development of immune-mediated and cancer diseases. RFB has been supported by János Bolyai Research Fellowship of the Hungarian Academy of Sciences.

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