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REVIEW ARTICLE

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The apoptosis cascade — morphological and immunohistochemical methods for its visualization

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Abstract Apoptosis is involved in morphogenesis of embryonic tissues as well as in homeostasis of adult organs and tissues. It is the main process by which organs maintain cell mass and at the same time eliminate excess and aged cells that have lost their functional importance. The typical morphological signs of apoptosis (cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation) are the final results of a complex biochemical cascade of events, some of which are inextricably linked to the process of differentiation. Studies that analyze all stages of this cascade, rather than the final morphological stages of apoptotic death, are essential in order that specific link(s) between differentiation and apoptosis are appreciated. This review outlines the main stages of the apoptosis cascade together with current methods for their morphological visualization. Starting with (a) receptors and ligands known to induce apoptosis, we continue with (b) early initiator stages of apoptosis, and (c) proteins regulating and potentially inhibiting further progression of the cascade, into (d) irreversible execution stages of the cascade, and finally (d) the morphological events of apoptotic death. For each stage we present those aspects of the biochemical background that are morphologically relevant, together with proven methods for their visualization. We offer technical advice at each stage based upon our experience of studying differentiation and apoptosis in human placental trophoblast.

Key words bcl-2 · Caspases · Differentiation · Phosphatidylserine flip · TUNEL

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Introduction

The term apoptosis was coined by Kerr et al. (1972) to describe morphologically distinct features of programmed, as opposed to accidental, cell death; the latter termed necrosis. These processes differ in their mechanisms of induction. Necrosis is induced by lethal chemical, biological or physical events. By contrast, apoptosis requires the coordination of gene-directed energy-dependent biological processes (Cotter et al. 1990). It is thus comparable to proliferation and differentiation and is usually initiated by specific receptor-ligand interactions. Apoptosis occurs if a cell is given sufficient time to organize a series of intracellular events that inevitably leads to its destruction. It therefore follows that the apoptosis cascade is under the intrinsic control of individual cells. Finally, apoptosis usually results in the formation of sealed cell fragments (apoptotic bodies), thus avoiding inflammation caused by the uncontrolled release of intracellular contents. Apoptosis can therefore be regarded as an injury-limiting mode of cell disposal. As a consequence of these complex mechanisms, apoptosis is characterized by specific structural alterations (such as blebbing of the cell surface, cell shrinkage, chromatin condensation and nuclear fragmentation) on a background of persistent integrity of the plasma membrane. It is thus clearly different from necrosis.

Apoptotic cell death involves the action of activators, effectors, and negative regulators. The process can be viewed as a cascade-like sequence of events, although the precise sequence of these events is not yet clear (Villa et al. 1997). It is likely that some steps in the apoptosis cascade differ between individual cell types, though this concept is not yet established. The complexity of the apoptosis cascade is further complicated by the fact that apoptosis is closely linked to the process of differentiation (Hengartner 1997). Both pathways partly use the same machinery. For example:

1. Involvement of caspases (known as the executioners during the apoptosis cascade) in lens fiber differentiation (Ishizaki et al. 1998)

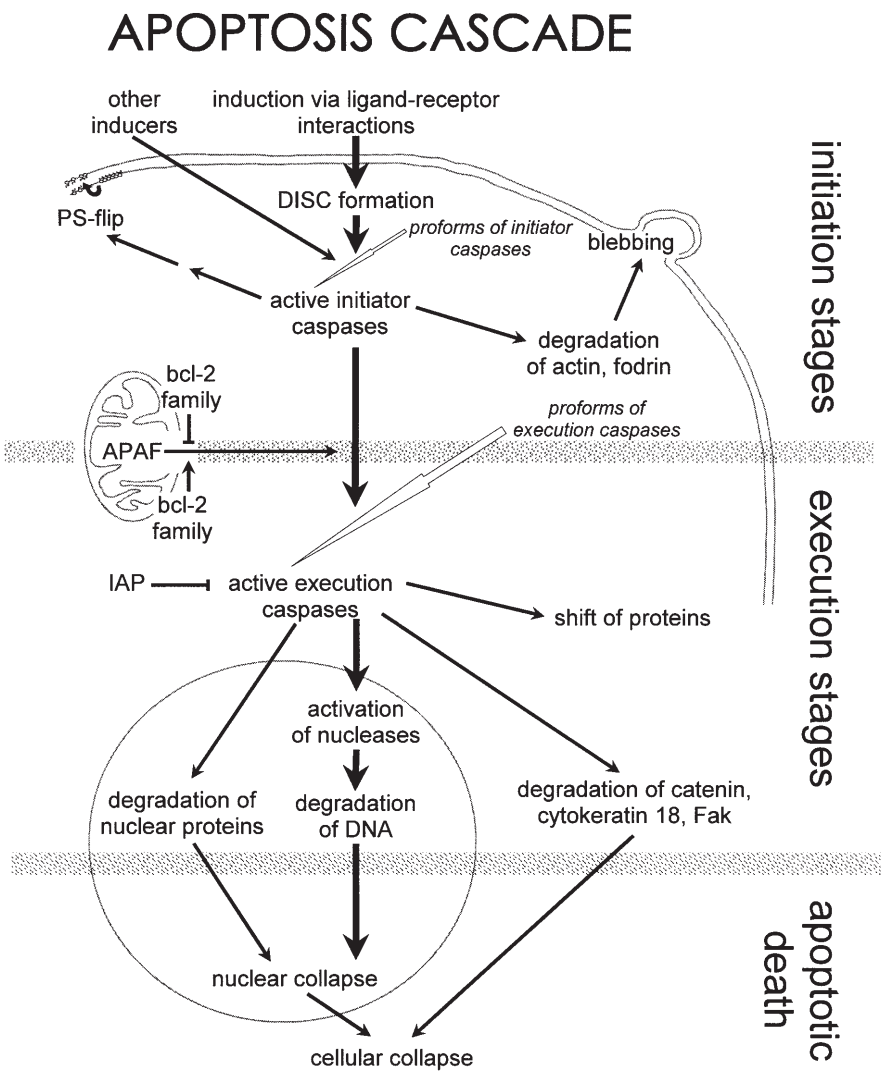


Fig. 1 The apoptosis cascade can be divided into three sets of sequential stages: *Initiation stages* include the induction of the cascade, e.g., by ligand-receptor interactions leading to first proteolytic events. *Execution stages* start with the activation of the execution caspases. Their activation is called the 'point of no return' since once activated, these proteases degrade a variety of proteins resulting in irreversible damage of the cell. *Apoptotic death* is the result of a very complex cascade of events that finally leads to the collapse of the nucleus and the cell itself. Even in this final stage the cell does not release intracellular components, thus avoiding inflammatory reactions

2. Cleavage of lamins as well as TUNEL reactivity (DNA cleavage) in terminal differentiation of erythroid cells (Morioka et al. 1998)
3. Apoptosis-induced phosphatidylserine flip during syncytial fusion resulting in the formation of placental syncytiotrophoblast (Huppertz et al. 1998)

Apoptosis was mainly regarded as a sequence of structural events until the early 1990´s. With increasing output of biochemical and molecular data over the past 5 years its cascade-character became more evident. An exponential increase in publications occurred during this time. The biomedical literature (Medline) before 1988 (16 years after the original definition of apoptosis) contains about 210 publications dealing at least in part with this subject. Between 1989 and 1993 a total of 1,480 apoptosis papers appeared and throughout the last 5 years, until fall 1998, a further 17,360 papers dealing with apoptosis were published. Though defined by an anatomist (Kerr et al. 1972), the anatomical interest in this process remains limited: we found only 112 contributions to apoptosis research among 4,800 papers published in seven leading morphological / histochemical journals throughout the last 5 years. Moreover, 83% of these studies were confined to the final stages of apoptotic death (annular chromatin condensation, TUNEL reactivity). Earlier events, such as the induction (4%), regulation (7%), and other stages of the apoptosis cascade (6%) were only rarely studied.

It is the intention of this review to highlight the significance, in cell biological terms, of the earlier stages of the apoptosis cascade. Our intention is to provide the reader with an integrated view – summarizing those methods that are available for the morphological visualization of each stage. In this way we hope to leave the reader with the general concept that studies of apoptosis that do not restrict themselves to the TUNEL test, but which consider earlier biochemical events and morphology, will advance knowledge of tissue dynamics. We have found this to be the case in our studies of apoptosis in the human placental trophoblast (Huppertz et al. 1998).

Overview of the apoptosis cascade

Tightly-controlled homeostatic mechanisms are required to regulate the balance between proliferative activity (increase in number of cells) and apoptotic activity (decrease in number of cells) in order that the functional and structural integrity of organs and tissues are maintained (Erickson 1997). Apoptosis is thus a highly regulated process that can be induced, stimulated and inhibited in different stages as follows (Fig. 1):

1. Apoptosis can be induced by specific ligand-receptor interactions. These include TNFα and its p55-receptor (TNF-R1), Fas-ligand (FasL) and its Fas-receptor (Fesus 1993).
2. Activation of these receptors leads to the formation of a signaling complex that subsequently activates a family of so-called initiator caspases, e.g., caspase 8 (Fraser and Evan 1996).
3. Initiator caspases are responsible for the first proteolytic events e.g. cleavage of cytoskeletal and related proteins including vimentin (van Engeland et al. 1997), actin (Kayalar et al. 1996), and fodrin (a membrane-associated cytoskeletal protein; Martin et al. 1995a; Greidinger et al. 1996). Amongst others, these early apoptotic events are thought to be responsible for the characteristic blebbing of the cell surface (McCarthy et al. 1997).
4. Cleavage of translocase (flippase) and/or activation of scramblase (floppase) leads to a subsequent flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. This is known to be a very early event during the initiation stages of apoptosis (Martin et al. 1995b). Externalization of phosphatidylserine is used by cells as a signal for events such as the attraction of macrophages, induction of the coagulation cascade or induction of syncytial fusion.
5. Initiator caspases are positioned at the top of the caspase hierarchy (Cohen 1997). Subsequently they cleave and thus activate a second subpopulation of caspases known as the execution caspases (Mignotte and Vayssiere 1998). Irreversible progression of the apoptosis cascade commences with activation of the latter.
6. Execution caspase activation is tightly regulated by the bcl-2 family of mitochondrial proteins. The various members of this family promote (bad, bak, bax, bcl-x_s, bik, hrk) or inhibit (A1, bcl-2, bcl-w, bcl-x_L, bfl-1, brag-1, mcl-1, NR13) cleavage of execution caspases (Hockenberry et al. 1990; Renvoize et al. 1997; Mignotte and Vayssiere 1998).
7. Irreversible progression of apoptosis by activated execution caspases (e.g., caspase 3) requires that these enzymes remain active for a critical minimum period of time (Marks et al. 1998). This varies from minutes to hours depending on the size, type and functional state of the cell.
8. Once activated, the execution caspases, either directly or by means of other proteases, cleave a broad array of proteins critical for cell survival. The latter include intermediate filament proteins (such as cytokeratin 18; Caulin et al. 1997), nuclear envelope proteins (such as lamins A and B; Greidinger et al. 1996), proteins involved in DNA maintenance and repair (such as poly-(ADP-ribose) polymerase (PARP); Tewari et al. 1995), enzymes involved in relaxation of the DNA-helix and separation of chromosomes during mitosis (topoisomerase IIα; Nakajima et al. 1996), the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{CS}; Song et al. 1996).
9. Execution caspases activate DFF (DNA fragmentation factor) and other endonucleases resulting in specific fragmentation of DNA (Liu et al. 1997).
10. Finally, execution caspases cause characteristic intracellular shifts and activation of proteins such as of: TIAR [T-cell-restricted intracellular antigen-related protein, possibly involved in cytoplasmic DNA cleavage (Taupin et al. 1995)], which is shifted from a nuclear to a cytoplasmic localization; transglutaminase II, which protects the endangered plasmalemma, by cross-linking proteins and forming large sub-plasmalemmal protein scaffolds (Cummings 1996; Fesus et al. 1996) that is shifted from a more diffuse cytoplasmic localization to directly underneath the plasma membrane.

In summary, the final apoptotic events comprise; down-regulation of transcription, fragmentation and condensation of DNA, alteration of nuclear and cellular shape, stabilization of the plasma membrane, and finally formation of apoptotic bodies (Kerr et al. 1995).

Stages of the cascade and morphological methods of visualization

Induction of apoptosis

Apoptosis can be induced by:

1. Ligands binding to respective receptors (e.g. FasL binding to Fas; TNFα binding to TNF-R1)
2. Cytolytics secreted by cytotoxic lymphocytes (e.g. granzyme B, perforin)
3. Disruption of cell-cell or cell-matrix interactions (e.g. disturbed interactions of cadherins or integrins with their ligands)
4. Presence (e.g. TGFβ) or absence (e.g. CSF) of specific growth factors
5. Increasing or decreasing levels of specific hormones (e.g. steroid hormones)
6. Non-physiological stimuli (such as serum starvation or irradiation)

Of the above list of initiation mechanisms, only receptor-ligand interactions have been studied at a morphological level in more detail.

Apoptosis-inducing receptors include those belonging to the TNF-receptor superfamily. This family comprises Fas (also known as Apo-1 or CD95), TNF-R1 (tumor

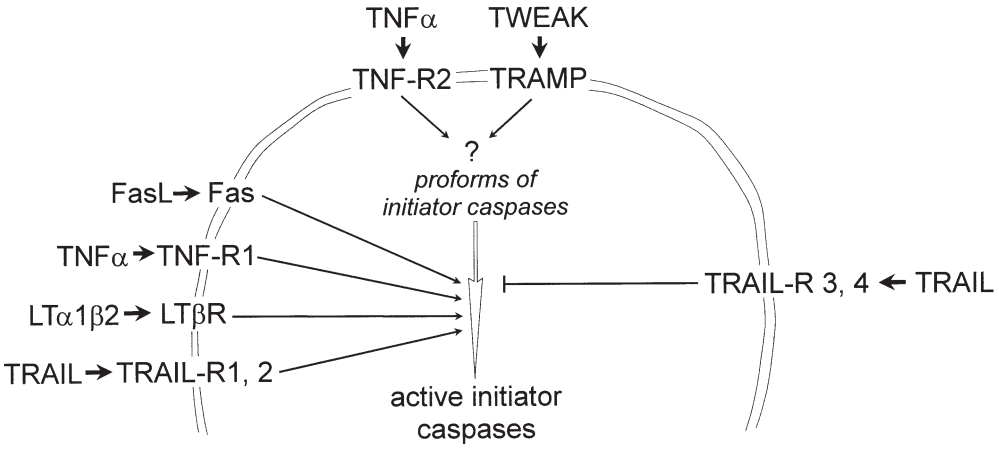


Fig. 2 Receptor-ligand interactions regulate the activation of initiator caspases. A variety of members belonging to the TNF-receptor superfamily are known to induce apoptosis by forming a death-inducing signaling complex (DISC); the latter activates members of the initiator caspases. At least one member of the TNF-receptor superfamily is known to block activation of initiator caspases; for some members the mode of action is still unknown

necrosis factor-receptor-1, p55 or CD120a) and other death receptors (TRAIL-R1/DR4, TRAIL-R2/DR5/Apo-2/TRICK2/KILLER, LTβR, CAR1, and DR6; Kidd 1998 and references therein; Pan et al. 1998; Hunt et al. 1999). These receptors share a specific intracellular domain, termed the death domain, activation of which triggers the apoptosis cascade (Fig. 2; for reviews see Yuan 1997; Kidd 1998). It is important to note that other receptors of the same superfamily (TRAIL-R3/DcR-1/TRID/LIT, TRAIL-R4/DcR-2/TRUNDD) act as so-called decoy receptors since they bind the same ligands but do not induce apoptosis (Hunt et al. 1999). It is still not clear if other members of this superfamily function as promoters or inhibitors of apoptosis (TNF-R2/CD120b, TRAMP/DR3/Apo3/WSL-1/LARD).

The most prominent ligands, Fas-ligand (FasL or CD95L) and TNFα, are type II membrane proteins expressed by the same, or neighboring, cells that induce apoptosis by autocrine or paracrine loops, respectively. Receptor binding can be accomplished either by cell-cell contacts or by ligand diffusion since both Fas-ligand and TNFα may be shed from the cell surface by enzymatic cleavage.

Methods and interpretation

Both ligand-receptor systems (Fas and its ligand FasL; TNF-R1 and its ligand TNFα) can easily be localized by immunohistochemistry since they are mostly present as integral membrane proteins. A large collection of antibodies is available to detect them – especially **Fas** and **FasL** (for antibody dilutions and putative sources see Table 1). In our hands, reliable immunohistochemical data can be obtained using cryostat sections following fixation with acetone or methanol (Huppertz et al. 1998); fixation with formaldehyde followed by embedding in paraffin often results in decreased immunoreactivity. Depending on tissue and mode of processing, clear immune reactions may be obtained in some cases following formalin-fixation and paraffin-embedding (Fig. 3a; Sasaki et al. 1998).

Immunohistochemical detection of **TNFα** and **TNF-R1** is possible using formalin-fixed paraffin sections (Fig. 3b).

Interpretation of these data may be problematic since immunohistochemical evidence of receptor expression in a given cell (and its ligand in the same or a neighboring cell) does not necessarily imply that the respective ligand/receptor interaction is involved in the induction of apoptosis. In the case of the Fas/FasL system, paracrine activation in most cases requires cell-cell contacts (Oyaizu et al. 1997). Autocrine activation requires cleavage of FasL by an as yet unknown metalloproteinase (Kayagaki et al. 1995); the latter is specifically required to enable the ligand to bind to the neighboring receptor. In the case of TNFα/TNF-R1, the situation is similar; however, paracrine activation over a certain distance by secreted/shedded TNFα seems to be a common phenomenon. In any case, proof for the functional importance of either system can only be obtained in vitro by induction of apoptosis in receptor-positive cells by the respective ligand.

Apoptosis may be induced experimentally in vitro using cells that express Fas or TNF-R1. The addition of either FasL or TNFα can be used reproducibly to study subsequent stages of the cascade in vitro (Cryns et al. 1996: FasL and TNFα; Katsen et al. 1998: TNFα; Simbulan-Rosenthal et al. 1998: FasL).

Initiator caspases

Although induction of apoptosis can be initiated via various interactions and stimuli, the final common pathway leads to activation of caspases (Table 2). This family of intracellular proteases cleaves their targets following aspartic acid residues (*c*ysteine *asp*art*ases*). At least 14 such proteases have been reported thus far (Miura et al. 1997), and the family is still growing. Phylogenetic comparisons of structural homology and sequence similarities leads to the division of the caspase-family into three subfamilies (Kidd 1998):

1. ICE-like caspases (including caspases 1, 4, and 5)
2. CPP32-like caspases (including caspases 3, 6, 7, 8, 9, 10)
3. ICH-1 subfamily (caspase 2)

In this review, we will confine our discussion to the CPP32-like caspases since convincing evidence exists for the participation of this subfamily in the apoptosis cascade (Miller 1997). Involvement of members of the other caspase subfamilies in the apoptosis cascade is currently controversial. As examples, different cell types are able to undergo apoptosis despite blocking/absence of caspase 1 (Casciola-Rosen et al. 1994; Nett-Fiordalisi et al. 1995; Schlegel et al. 1996), and caspase 2 appears to be involved in neuronal apoptosis (Stefanis et al. 1998)

Table 1 Markers of apoptosis and tools for their morphological visualization as tested in our laboratory. The list of putative sources is incomplete; a variety of other companies distributes tools for morphological investigation of apoptosis

Protein/event	Tools	Putative source
Fas/FasL	Various antibodies	Upstate Biotechnology R&D Systems
TNF-R1/TNFα	Various antibodies	R&D Systems
Initiator caspases		
Caspase 8	Antibodies against proform Coloured substrates for ELISA	Santa Cruz Biotechnology Clontech
Caspases 8, 9, 10	Antibodies against pro- and active forms	Santa Cruz Biotechnology
Cytoskeletal proteins, e.g. Fodrin	Various antibodies	ICN
Membrane blebbing	Light and electron microscopy	–
Phosphatidylserine flip	Annexin V	Clontech
bcl-2 Family of proteins	Various antibodies	Sigma Upstate Biotechnology
Execution caspases		
Caspase 3	Antibodies against proform Coloured enzyme-substrates (viable cells)	Upstate Biotechnology OncoImmunin (PhiPhiLux)
	Coloured enzyme-substrates (for ELISA, only)	Clontech
Caspases 3, 6, 7	Antibodies against pro- and active forms	Transduction Laboratories Santa Cruz Biotechnology
PARP	Various antibodies	Upstate Biotechnology
Lamin B	Various antibodies	Oncogene/Calbiochem
Caspase-cleaved cyto keratin 18-fragments	Monoclonal antibody	Boehringer Mannheim
TIAR	Various antibodies	Transduction Laboratories
Transglutaminase II	Various antibodies	Upstate Biotechnology
DNA single strand breaks	TUNEL	Calbiochem Enzo Diagnostics
	Monoclonal antibody to ssDNA	Alexis Corporation
Annular chromatin condensation	Light and electron microscopy	–

but not in the programmed cell death of B-chronic lymphocytic leukemia cells (King et al. 1998).

According to their role in the apoptosis cascade, the CPP32-like caspases are classified as either initiator or execution caspases (Fraser and Evan 1996; Mignotte and Vayssiere 1998). For nomenclature and abbreviations of caspases, see Table 2.

The initiator caspases (caspases 8, 9 and 10; Fraser and Evan 1996) are activated by the inducers of apoptosis, e.g., Fas or TNF-R1 (Figure 2). Upon activation by their ligands FasL and TNFα, respectively, a death-inducing signaling complex (DISC) is formed and binds to the activated receptor. The precise interactions between proteins forming this complex remain to be elucidated. However it appears that there are several redundant pathways for the initiation of apoptosis (for review see Kidd 1998). Proteins involved in this complex, such as the Fas-associated death domain protein (FADD), activate initiator caspases such as caspase 8 by binding to the death domain, and subsequent cleavage of the prodomain of the protease results.

Methods and interpretation

Antibodies for immunohistochemistry are available for *procaspase 8*; and antibodies recognizing specifically the *active and procaspases 8, 9 and 10* can be obtained (Table 1). According to our experience these antibodies can be applied to formalin-fixed paraffin sections without any pretreatment (Fig. 3c). For enzyme histochemical proof of caspase activity (caspase 8) respective substrates are available (Table 1). However these dyes require ELISA detection and thus can only be used with cell lysates rather than on viable cells or on tissue sections.

The inactive proforms of the caspases consist of single molecules containing a prodomain and two catalytic subunits. Activation of the caspases is achieved by proteolytic removal of the prodomain and furthermore by proteolytic separation of the two catalytic subunits. Subsequently the latter aggregate to generate the heterodimeric active enzyme (Thornberry 1996). Currently available antibodies recognize a sequence in the prodomain and there-

fore recognize only the inactive proforms – they cannot recognize activated caspases. Antibodies directed against a sequence within a catalytic subunit will recognize both the inactive proform and the active form of the caspase. Currently no antibodies exists that specifically recognize the heterodimeric active enzyme. This limitation makes immunohistochemical interpretation of caspase activation difficult at the present time.

One approach we have developed to overcome this problem is to look for co-localization of procaspase 8 with one of the apoptosis receptors (Fas or TNF-R1). Such evidence suggests biological activity (Fig. 3a–c). A less convincing indirect method of studying activation of these caspases is to subtract the immunoreactivities, on serial sections, obtained using antibodies recognizing either pro and active enzyme, or pro-enzyme alone.

Table 2 Caspases, their names and substrates. Data are derived from Alnemri 1997, Cohen 1997, Miller 1997, Miura et al. 1997, Villa et al. 1997, and Kidd 1998 [*CED Caenorhabditis elegans* cell death protein, *CMH-1* CPP32/Mch2 homolog, *CPP32* cysteine protease protein with a molecular weight of 32 kDa (caspase 3), *DNA-PK*$_{cs}$ catalytic subunit of the DNA-dependent protein kinase, *FADD* Fas-associated death domain protein, *FLICE* FADD-like ICE, *ICE* interleukin-1β-converting enzyme (caspase 1), *ICE*$_{rel}$ ICE-related protease, *ICE-LAP* ICE-like apoptotic protease, *ICH* ICE/CED-homolog, *IL-1*β interleukin-1β, *MACH* MORT1-associated CED-3 homolog, *Mch* mammalian CED homolog, *MORT* mediator of receptor-induced toxicity, *PARP* poly(ADP-ribose) polymerase, *PKC*δ protein kinase Cδ, *SREBP* sterol regulatory element binding protein, *TX* transcript X (caspase 4), *TY* transcript Y (caspase 5), *U1–70 kDa* 70 kDa protein component of the U1-small ribonucleoprotein]

Caspase	Old names	Subfamily	Substrates I (other proteins)	Substrates II (other caspases)
Caspase-1	ICE	ICE-like	Pro-IL-1β, PARP, actin	Pro-caspases 1, 2, 3, 4,
Caspase-2	ICH-1, Nedd-2	ICH-1-like	PARP (?)	Pro-caspase 2
Caspase-3	CPP32, Yama, apopain	CPP32-like, execution casp	PARP, DNA-PK$_{cs}$, SREBP, U1–70 kDa PKCδ, actin	Pro-caspases 2, 3, 6, 7, 9
Caspase-4	ICH-2, ICE$_{rel}$II, TX	ICE-like	PARP, U1–70 kDa, DNA-PK$_{cs}$	Pro-caspases 1, 3, 4
Caspase-5	ICH-3, ICE$_{rel}$III, TY	ICE-like	?	?
Caspase-6	Mch2	CPP32-like, execution casp	Lamins A, B1, C, (PARP)	Pro-caspases 3, 6, 7
Caspase-7	Mch3, CMH-1, ICE-LAP3	CPP32-like, execution casp.	PARP, U1–70 kDa, SREBP	Pro-caspases 6, 7
Caspase-8	Mch5, MACH1, FLICE1	CPP32-like, initiator casp.	PARP, fodrin[a]	Pro-caspase 1-like, Pro-caspase 3-like, Pro-caspase 8-like
Caspase-9	Mch6, ICE-LAP6	CPP32-like, initiator casp.	PARP, fodrin[a]	Pro-caspase 3
Caspase-10	Mch4, FLICE2	CPP32-like, initiator casp.	PARP, U1–70 kDa, fodrin[a]	Pro-caspase 1-like, Pro-caspase 3-like, Pro-caspases 8, 10

[a] Fodrin cleavage has been reported to take place before activation of execution caspases (Cryns et al. 1996, Greidinger et al. 1996), not specifying which initiator caspase is responsible for cleavage

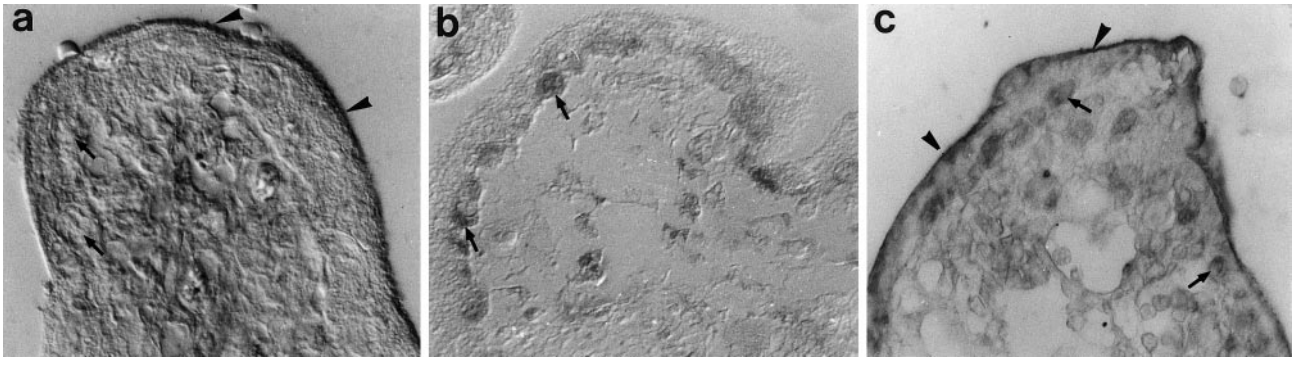


Fig. 3a–c Immunohistochemical reaction patterns of proteins involved in the initiation stages of apoptosis. **a** The Fas receptor is present along the apical surface of the syncytiotrophoblast (*arrowheads*) covering placental villi. No reactivity can be found in the underlying trophoblastic stem cells (*arrows*). **b** In contrast, the TNF-receptor-1 shows immunoreactivity only in the stem cells (*arrows*). **c** One member of the initiator caspases, caspase-8, is known to be activated by both receptors, Fas and TNF-R1. The inactive proform can be localized in the direct vicinity of both of the above receptors, beneath the apical plasma membrane of the syncytiotrophoblast (*arrowheads*) and in the underlying stem cells (*arrows*). This distribution pattern suggests that apoptosis can be induced in both layers of the trophoblast. ×800

Early proteolytic events

The initiator caspases are responsible for most of the early proteolytic events in the course of apoptosis. However, participation of parallel pathways that bypass the initiator caspase complex, (apoptosis induced by mechanisms other than receptor-ligand interactions) cannot be ruled out (Thompson 1998). Early proteolytic events comprise cleavage of fodrin (an abundant membrane-associated cytoskeletal protein; Cryns et al. 1996), vimentin (van Engeland et al. 1997) and actin (Kayalar et al. 1996).

Blebbing

Early proteolytic events of the initiator caspases and the resulting cleavage of cytoskeletal and related proteins leads to shrinkage of the cell and blebbing of the cell surface results. Modification of cytoplasmic proteins in association with apoptosis events, e.g. phosphorylation of myosin, are also involved in the formation of membrane blebs (Mills et al. 1998).

Methods and interpretation

Immunohistochemical proof for enzymatic cleavage of actin, fodrin, and vimentin is difficult to obtain; reliance upon loss of immunoreactivity is unacceptable and therefore for the present time one can only hint at their degradation by light microscopical or ultrastructural detection of cellular *blebbing* (Fig. 4).

It is important to appreciate that cellular blebbing can be induced by factors other than apoptosis, especially osmotic shock (Gass and Chernomordik 1990). Moreover, as demonstrated by Laster and Mackenzie (1996), bleb formation occurs during mitosis. Fortunately these types of blebbing can be distinguished from each other: f-actin is present inside the blebs during mitosis while f-actin is restricted to the bases of blebs during apoptosis (Laster and Mackenzie 1996). Due to these difficulties in determining the cause of membrane blebbing, this morphological event is not sufficient, in isolation, to assign the state of apoptosis in a cell. This caveat is demonstrated by conflicting in vitro results: McCarthy et al. (1997) found blebbing as a very early event in apoptosis before the onset of nuclear changes whereas Katsen et al. (1998) showed that formation of blebs only appeared in cells with condensed chromatin, i.e., during late stages of apoptosis.

Phosphatidylserine flip

The flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane is another early apoptosis event (Martin et al. 1995b). Aminophospholipids such as phosphatidylserine or phosphatidylethanolamine are normally confined to the inner leaflet of the plasma membrane; while the outer leaflet contains mainly neutral phospholipids such as phosphatidylcholine (Bevers et al. 1996). This asymmetry in membrane aminophospholipid composition is achieved by the activity of enzymes such as the ATP-dependent aminophospholipid translocase (also termed ‘flippase’) that preferentially transports phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet (Williamson and Schlegel 1994). Active translocase counteracts spontaneous flipping of phosphatidylserine to the outer leaflet by relocating it to the inner leaflet. In the early stages of apoptosis, activation of the initiator caspase complex results in inversion of the direction of the translocase activity (Obringer et al. 1997) or in activation of translocating proteins (scramblases, also termed ‘floppases’) that transport aminophospholipids in a reverse manner (Zwaal et al. 1993). The final consequence of either event is the accumulation of phosphatidylserine in the outer membrane leaflet (phosphatidylserine flip) (Fig. 5).

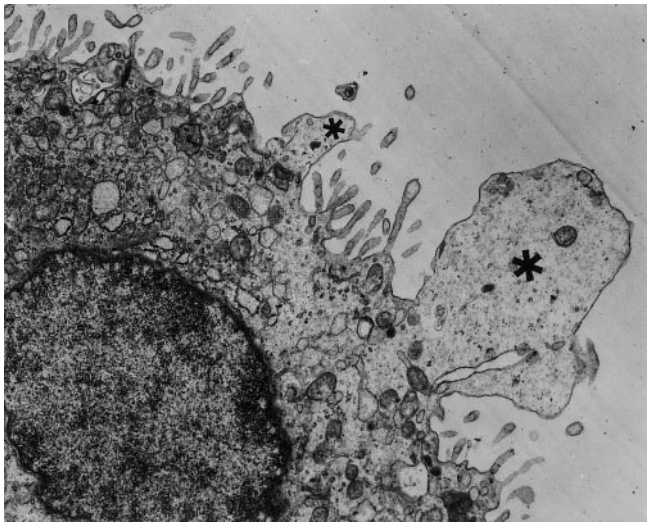


Fig. 4 Formation of blebs (*) at the apical membrane of the syncytiotrophoblast. In contrast to the syncytioplasma beneath, the blebs are characterized by a paucity of cellular organelles. The nucleus does not show any signs of chromatin condensation, indicating that bleb formation is an early event during apoptosis. ×8000

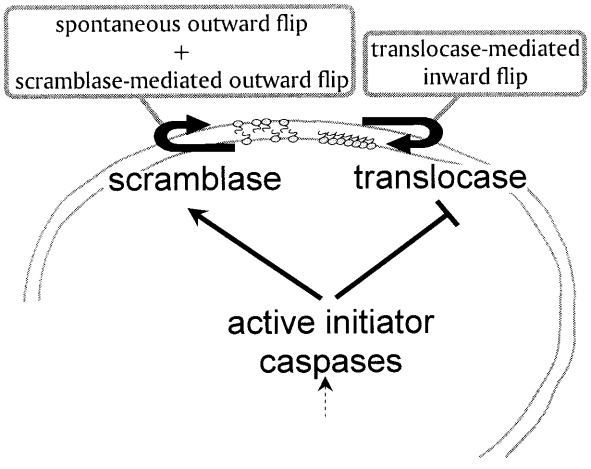


Fig. 5 Mechanisms regulating the distribution of phosphatidylserine (PS) in the two leaflets of the plasma membrane. In normal cells, PS is kept in the inner leaflet of the plasma membrane by the activity of ATP-dependent translocases. During early stages of apoptosis, the initiator caspases lead to the inactivation of translocases and/or to the activation of scramblases. This results in a flip of PS from the inner to the outer leaflet

Promotion of the apoptotic phosphatidylserine flip (phosphatidylserine flip as an active process – Martin et al. 1995b) serves as a signal for a variety of mechanisms such as syncytial fusion (Lyden et al. 1993; Adler et al. 1995), signaling for cell-cell recognition, and induction of the coagulation cascade (Bevers et al. 1996). Furthermore, it has been described as an 'eat-me' signal (Savill 1998) by apoptotic cells in order that they are recognized by phagocytes.

Methods and interpretation

Translocase- or scramblase-antibodies are not yet available. Thus microscopical visualization of this process requires direct proof of the *phosphatidylserine flip* itself. This is possible by application of phosphatidylserine antibodies or of annexin V which specifically binds to this particular phospholipid (Thiagarajan and Tait 1990; Koopman et al. 1994). However by implication these methods are not applicable on tissue sections since antibodies and annexin V will bind phosphatidylserine residues present either in the outer or the inner plasmalemmal leaflet. Plasmalemmal staining thus occurs in the absence of a phosphatidylserine flip. Because of this, reliable histochemical proof of the flip at present is restricted to the use of intact cells (incubation of isolated vital cells with FITC-coupled annexin V or with PS-antibodies). In order to avoid false-positive identification of a phosphatidylserine flip in damaged cells with leaky membranes (which would allow annexin V-penetration into the inner leaflet), kits have been developed which combine annexin V and propidium iodide (PI). The latter is a nucleic acid stain which cannot permeate the intact plasma membranes and thus annexin V staining can be taken as evidence of phosphatidylserine flip in the absence of nuclear PI staining. Normally these kits are applied to isolated cells, but with some caution they also give meaningful data (Fig. 6) when applied to tissue explants studied by confocal microscopy (Huppertz et al. 1998).

Four different results can be obtained when applied to cells or tissue explants:

1. Cells which are neither stained by annexin V nor by PI are likely to be intact, not having entered the apoptosis pathway.
2. Presence of annexin V-binding to the plasmalemma with absence of nuclear staining with PI points to early stages of apoptosis with phosphatidylserine flip.
3. Plasmalemmal annexin V-staining together with annular PI-staining of the nucleus is characteristic for final stages of apoptosis with nuclear condensation and increasing membrane permeability.
4. Finally, diffuse annexin V-positivity combined with diffuse cytoplasmic and/or nuclear PI-staining suggests non-apoptotic membrane damage (e.g. necrosis).

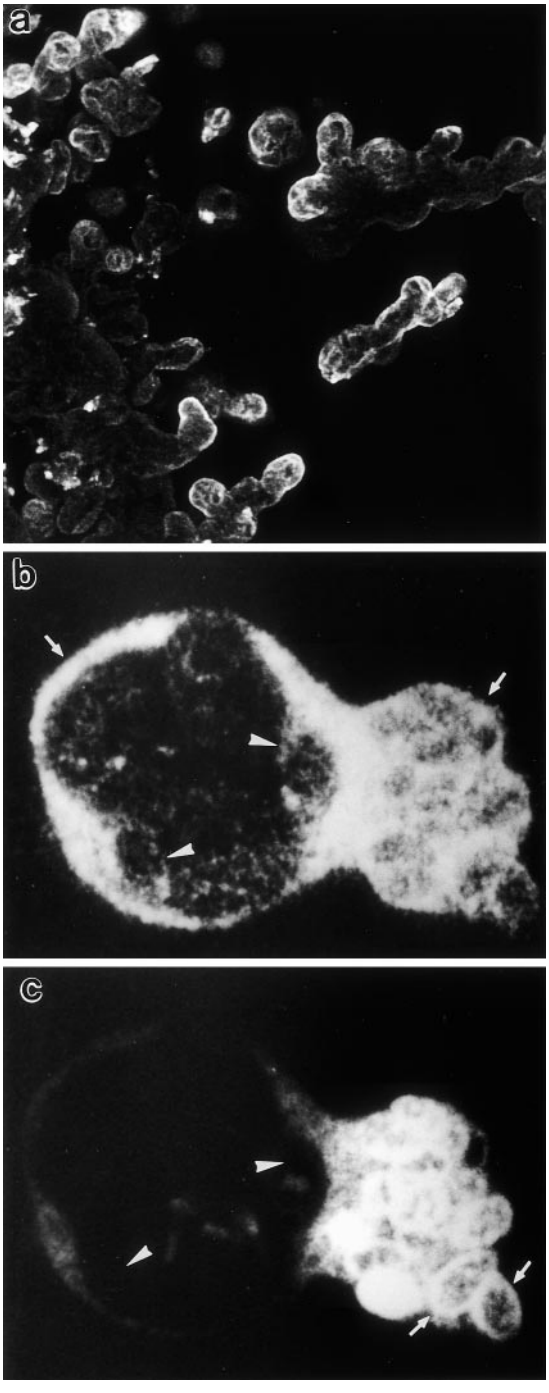


Fig. 6a–c Visualization of the PS-flip in a tissue using confocal laser microscopy. Explants of placental villi were incubated with FITC-coupled annexin V and propidium iodide. In **a** the villi can be seen with focal annexin V-labeling of the villous surface. A higher magnification **b** in the region of a syncytial sprout (area where apoptotic nuclei are extruded) demonstrates annexin V-staining of the syncytiotrophoblast (*arrows*) and also faint staining of the plasma membrane of some of the underlying trophoblastic stem cells (*arrowheads*). In **c** staining with propidium iodide is shown in the identical sectional plane. Propidium iodide does not cross intact membranes. The nuclei in the syncytial sprout are clearly positive, partly demonstrating annular chromatin condensation typical for late stages of apoptosis (*arrows*). The trophoblastic stem cells which showed annexin V-positivity, do not reveal any PI-staining (*arrowheads*). The combination PI-positivity with annexin V-binding (sprout) points to late apoptotic stages; the combination PI-negativity with membranous annexin V-binding (trophoblast cells) indicates PS-flip during initiation stages of apoptosis. **a** ×105; **b, c** ×1120

Inhibition and promotion

The two early apoptotic events discussed, namely alterations of the cytoskeleton (cleavage of cytoskeletal proteins leading to blebbing) and of the plasma membrane (phosphatidylserine flip) are both induced by the initiator caspases. However they are not necessarily followed by

immediate execution of apoptosis and death of the cell (McCarthy et al. 1997). Rather, before entering the irreversible stages, the cascade can be blocked or delayed by inhibiting activation of the execution caspases (caspases 3, 6 and 7; Mignotte and Vayssiere 1998; Fig. 1). Normally, activation of their proforms takes place via cleavage by the initiator caspases. This critical step is controlled by members of the bcl-2 family and related mitochondrial proteins. These may either partially inhibit, or promote, further activation of execution caspases by initiator caspases. In mammals, at least 15 distinct gene products of the bcl-2-family have been identified: A1, bcl-2, bcl-w, bcl-x_L, bfl-1, brag-1, mcl-1 and NR13 are inhibitors of apoptosis while bad, bak, bax, bcl-x_S, bid, bik, hrk, and mtd promote apoptosis (Fig. 7; for review see Kroemer 1997; Kidd 1998; Inohara et al. 1998).

The mechanisms whereby members of the bcl-2-family effect inhibition/stimulation of the execution caspases is still under debate. Different models of direct or indirect actions have been proposed:

1. Homodimerization of stimulatory members may promote apoptosis whereas heterodimerization of inhibitory and stimulatory members appears to have the opposite effect (Oltvai et al. 1993; Sedlak et al. 1995).
2. Recent studies by Hsu and Youle (1997, 1998) demonstrated that the ability to form dimers depends on the presence of detergents; no dimers were formed in the absence of detergents. Moreover, during apoptosis the preferentially cytoplasmic members of the bcl-2 family (e.g., bax, bcl-x_L) are redistributed to the mitochondrial membranes where they act respectively as agonists or antagonists of apoptosis (Hsu et al. 1997).
3. Another model suggests that the members of the bcl-2 family control release of proforms of execution caspases from the mitochondria into the cytoplasm whereupon activation takes place by initiator caspases (Mancini et al. 1998; Samali et al. 1998).
4. Finally, recent data suggest that the bcl-2 family controls release from the mitochondria into the cytoplasm of other apoptosis-promoting molecules (Fig. 7; Kluck et al. 1997; Rosse et al. 1998). Alternatively the bcl-2 family may directly interact with these promoting molecules (Hu et al. 1998). These ‘apoptotic protease activating factors’ (apaf) participate in the initiator caspase-dependent activation of execution caspases (Cecconi et al. 1998). During apoptosis cytochrome-c (apaf-2) is released from mitochondria and binds to apaf-1, a Ced-4 homolog, which in turn binds via its CARD domain (caspase activation and recruitment domain) to caspase 9. Finally caspase 9 activates caspase 3 (Zou et al. 1997; Chou et al. 1998).

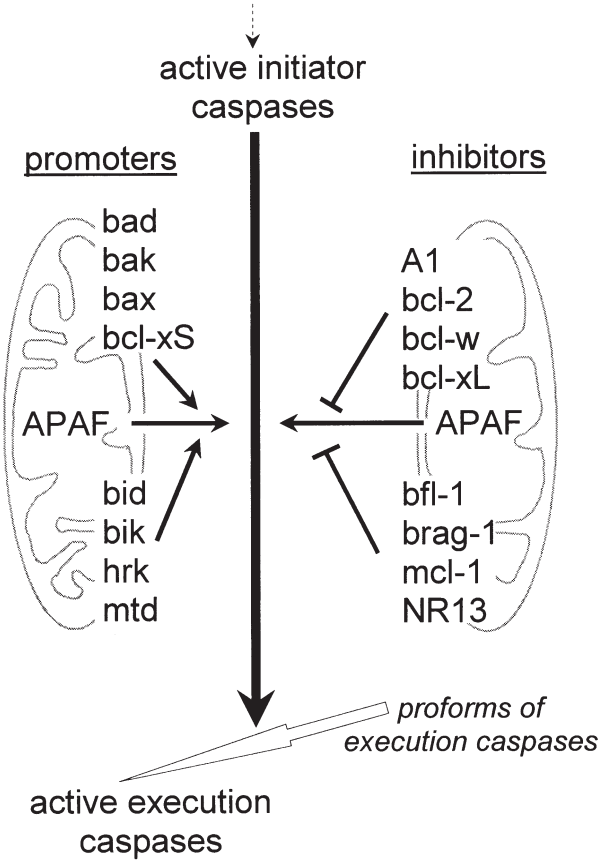


Fig. 7 Mitochondrial regulation of activation of execution caspases. The ‘hallmark of apoptosis’ is tightly regulated by a variety of proteins belonging to the bcl-2 family. On the left, we have listed the promoters furthering the activation of execution caspases by initiator caspases. On the right, the inhibitors are listed that lead to the stopping or to a prolongation of the cascade

Though the underlying mechanisms are unclear, the inhibitory effects of bcl-2 and mcl-1 are of paramount importance for the regulation of apoptosis.

Bcl-2 and Mcl-1

These inhibitors of apoptosis have been detected in a broad variety of cells and tissues in which apoptosis can be temporarily inhibited in vitro (e.g., T lymphocytes; van Parijs et al. 1998). In many tissues immunolocalization of bcl-2 and mcl-1 can be demonstrated in proliferating stem cells whereas the differentiating subsets that leave the cell cycle show decreasing expression of these molecules (Bonkhoff et al. 1998). Yang et al. (1995) have described a differential expression pattern of both inhibitors: In ML-1 cells (a human myeloblastic leukemia cell line that can differentiate to monocytes and macrophages) mcl-1 expression is minimal during proliferation and reaches a maximum only during early differentiation. By contrast bcl-2 expression is maximal in immature proliferating ML-1 cells and declines slowly during differentiation. These data support a concerted action for these inhibitors: throughout the cell cycle apoptosis can be continuously prevented by bcl-2, which has a rather long half-life of 10–14 h. During the transitional period to differentiation, mcl-1, an inhibitor with a short half-life (1–3 h), seems to allow a more flexible regulation. Later differentiation is governed by declining

amounts of bcl-2 and finally results in apoptosis. Differential half-lives of these inhibitors can be seen as a pathway for stable versus flexible inhibition of apoptosis (Yang et al. 1995).

Our own data on bcl-2 and mcl-1 expression in proliferating and differentiating human placental trophoblast suggests that the above interpretation may be tissue-specific. In placental trophoblast the stem cells (villous cytotrophoblast) initiate early apoptotic mechanisms (initiator caspase-induced phosphatidylserine flip) necessary for the induction of syncytial fusion. However the simultaneous concerted action of both of these inhibitors prevents newly-fused trophoblast from irreversibly proceeding along the apoptosis cascade (Huppertz et al. 1998).

Methods and interpretation

A large number of antibodies recognize the various members of the *bcl-2 family* facilitating their immunohistochemical localization. In our experience cryostat sections fixed in absolute acetone give reproducible information, whereas with paraffin sections the duration of formalin fixation seems to be crucial. However groups working with obviously identical methods have obtained clearly contrasting data with the same tissues. Until consistent data are obtained, immunohistochemistry of these molecules and its interpretation should be viewed with caution. This is of particular importance since inhibition or progress of the apoptosis cascade depends on the ratio of agonistic and antagonistic members of the bcl-2 family. Simple detection of only one of these components may be difficult to interpret and dual-immunohistochemistry may be required to advance our knowledge of the bcl-2 family.

Inhibitors of Execution Caspases (IAP)

A new family of inhibitors has recently been described in addition to the bcl-2 family of apoptosis regulators. Known as ‘inhibitors of apoptosis proteins’ (IAP) they represent direct inhibitors of execution caspases (caspases 3, 6, 7) rather than of initiator caspases (caspase 8; Deveraux et al. 1997, 1998; Roy et al. 1997). Methods for the morphological visualization of their expression and action have not, to our best knowledge, yet been described.

Execution caspases

According to the present state of knowledge, apoptotic death is irreversibly induced once the execution caspases are activated. Their activation leads to the degradation of a variety of proteins and to the activation of degrading enzymes, e.g. nucleases. Therefore, the activation of the execution caspases is termed the hallmark of apoptosis – the point of no return leading to execution of cell death. The general validity of this view has however been challenged by recent experiments: Short term activation of caspase 3 in vitro, followed by its inactivation within a few hours, did not result in apoptotic death (Marks et al. 1998). The allotted time interval for activation of caspase 3 without execution to death is in the range of hours and seems to increase with the size of the cell. Thus the physiologic relevance of these data remains uncertain at the present time.

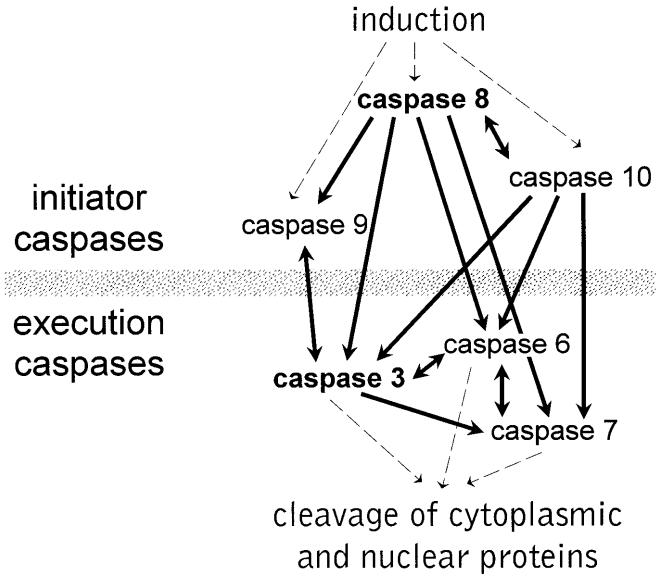


Fig. 8 The functional interrelations between the two groups of CPP32-like caspases, initiator and execution caspases are shown. In addition to the activation steps shown in this scheme, self-activation of different caspases is also known

Caspases 3, 6, 7

The closest mammalian homolog of the *Caenorhabditis elegans* CED-3 protein is caspase 3 (also known as CPP32, YAMA or apopain; Fernandez-Alnemri et al. 1994) from which the term caspase 3-subfamily (CPP32-like caspases) was derived. Caspase 3 was found to degrade a variety of cytoplasmic and nuclear proteins (Table 2) and to activate nucleases – thereby inducing degradation of DNA. Localization within the cell is not yet clear. According to recent experiments the inactive proform is localized in both the mitochondria and cytoplasm whereas the active form is located in the cytoplasm and the nucleus (Mancini et al. 1998; Samali et al. 1998).

Caspases 6 and 7 act as execution caspases downstream to the initiator caspases (Fig. 8; Lincz 1998). In some systems caspase 6 was found to be activated prior to caspase 3 (Grossmann et al. 1998); in others caspase 8 activates caspase 3, which in turn activates caspase 7, leading finally to the activation of caspase 6 (Cohen 1997).

Methods and interpretation

As previously discussed for the initiator caspases, antibodies are available that recognize either the proform or both the pro- and the

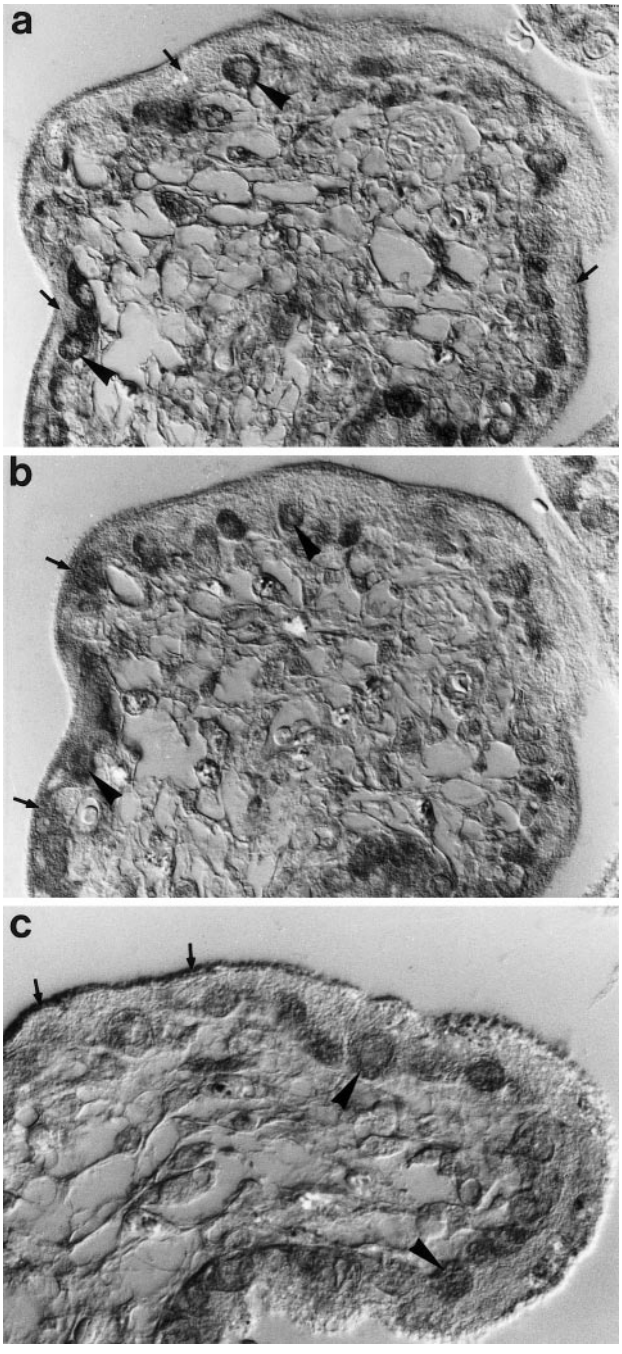


Fig. 9a–c Interpretation of immunohistochemical reaction patterns of proteins involved in the execution stages of apoptosis. **a** The inactive proform of caspase 3 is found predominantly in the stem cells of the villous trophoblast (*arrowheads*) whereas the syncytiotrophoblast is mostly devoid of immunoreactivity (*arrows*). **b** The antibody recognizing both the inactive proform and the active form of caspase 3 shows not only reactivity in the stem cells (*arrowheads*) but also focal reactivity within the syncytiotrophoblast (*arrows*). This suggests focal activation of caspase 3 only in the syncytiotrophoblast. **c** Focal activation of execution caspases within the syncytiotrophoblast can be visualized by a shift of transglutaminase II into a subplasmalemmal position (*arrows*); in the trophoblastic stem cells, transglutaminase II is evenly distributed throughout the cytoplasm (*arrowheads*) indicating absence of caspase 3-activation. ×800

active forms of *caspase 3*. These antibodies can be applied to formalin-fixed paraffin sections without any pretreatment. However since neither specifically recognizes the active form they cannot be used to infer caspase 3 activation. Careful comparison of antibodies directed against the proform with those directed against pro- and active forms, may point to sites of activation: in human placental villous trophoblast, immunoreactivity for pro-caspase 3 predominates in the proliferating stem cells (mononuclear villous cytotrophoblast) whereas the highly differentiated stage of villous trophoblast, the syncytiotrophoblast is preferentially stained by the antibody binding to both the pro- and the active forms of the enzyme (Fig. 9a, b; Huppertz et al. 1998). These data suggest that activation of caspase 3 in the trophoblast is confined to the syncytiotrophoblast.

A novel approach may be used to overcome this limitation of immunohistochemistry. New synthetic dyes have been developed that provide histochemical proof of enzyme activity for several caspases (Table 1). One such substrate, for caspase 3 (PhiPhiLux), can be used on viable intact cells. The method relies on the fact that caspase 3 cleavage of PhiPhiLux produces fragments that accumulate intracellularly because their rate of diffusion through intact membranes is significantly slower than for the intact substrate. We have used PhiPhiLux to demonstrate that caspase 3 activity is present in isolated mononuclear fragments of syncytiotrophoblast but not in the villous cytotrophoblast stem cells (Fig. 10a, b). Preliminary attempts in our laboratory to demonstrate cell specificity of caspase 3 activity by this method on formalin-fixed paraffin-embedded tissue sections are encouraging. By contrast this approach appears to be less rewarding with unfixed cryostat sections due to loss of tissue section integrity, washing and diffusion artifacts. Other caspase substrates can only be used with cell lysates where activity is demonstrated by Elisa measurements (Table 1).

Antibodies are available to detect *caspases 6 and 7*, but thus far these cannot distinguish between pro- and active forms of these caspases (Table 1).

Apoptotic cleavage of proteins, resulting in their degradation

In similarity with the initiator caspases, execution caspases were found to cleave a variety of cytoplasmic and membrane proteins, e.g., (Fig. 11):

1. α- and β-Catenin (by caspases 3 and 6; Brancolini et al. 1997; Herren et al. 1998) forming intracellular links between cadherins and actins
2. Fak, focal adhesion kinase (by caspases 3 and 6; Wen et al. 1997; Gervais et al. 1998) promoting contacts between cell and extracellular matrix
3. The intermediate filament cytokeratin 18 (by caspases 3, 6, 7; Caulin et al. 1997)

However the principal targets of this group of caspases reside within the nucleus (Figs. 11, 12) and comprise:

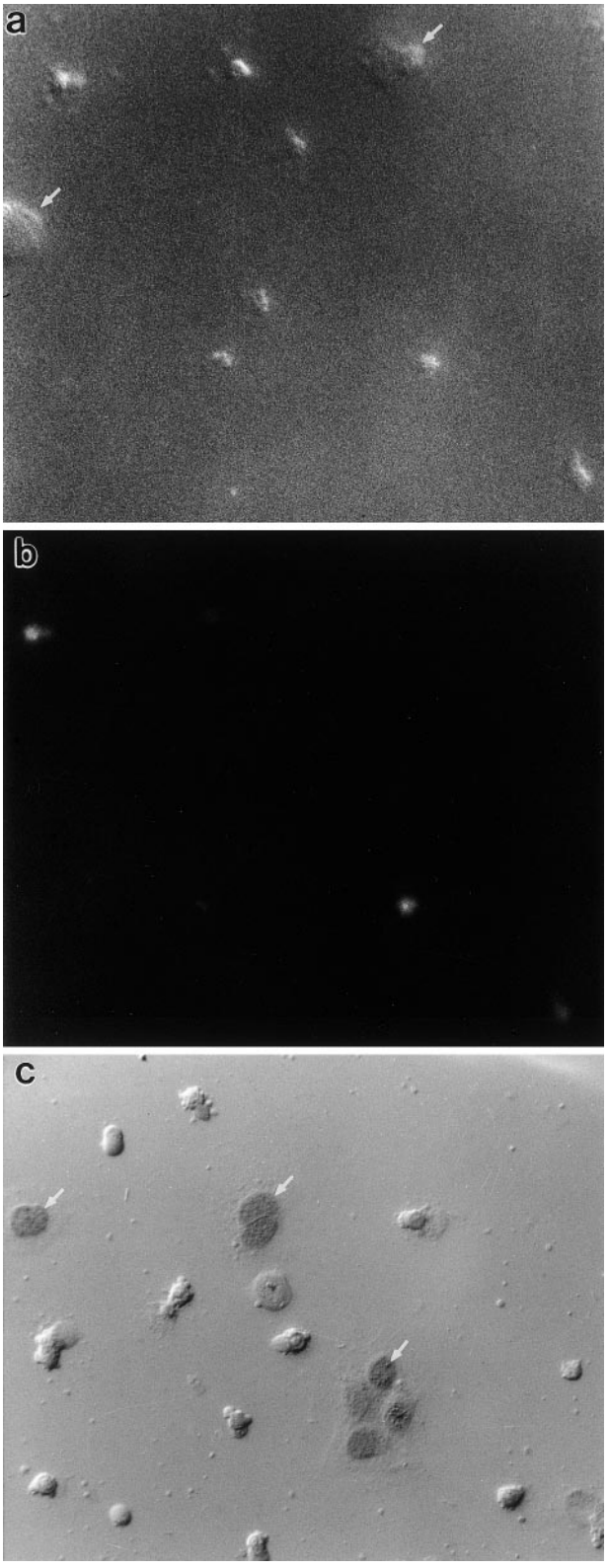
1. Poly-(ADP-ribose)polymerase (PARP)
2. Nuclear envelope proteins; lamin A, lamin B, and lamin C
3. The 70 kDa protein component of the U1 small nuclear ribonucleoprotein (U1–70 kDa)
4. The catalytic subunit of the DNA-dependent protein kinase ($DNA\text{-}PK_{CS}$)

These targets are not uniformly cleaved by all execution caspases; rather each caspase has a different spectrum of targets, which partly overlap with each other (Table 2; Alnemri 1997; Miller 1997; Miura et al. 1997; Villa et al. 1997; Kidd 1998).

PARP and lamins

Poly-(ADP-ribose)polymerase (PARP) catalyzes ADP-ribosylation of nuclear proteins at the sites of spontaneous DNA strand breaks. PARP thus facilitates the repair of sites of DNA damage. Furthermore, PARP inhibits Ca^{2+}/Mg^{2+}-dependent endonucleases that cleave DNA during apoptosis (Tanaka et al. 1984). Clearly PARP is important for the maintenance of DNA integrity, and it is not surprising that PARP is one of the targets for a variety of caspases (Table 2); cleavage results in reduced DNA repair and therefore increased damage to chromosomes (Fig. 12).

Lamins are the major structural proteins of the nuclear envelope and are found along the inner surface of the nuclear membrane. Here they are involved in maintaining the structure of the nucleus. Their apoptotic degradation leads to structural changes in the nucleus, culminating in its collapse and fragmentation (Fig. 11). Lamins are generally cleaved by caspases other than caspase 3, principally caspase 6 (lamin A, Takahashi et al. 1996; lamin B, Cuvillier et al. 1998). During apoptosis cleavage of lamin B occurs before that of lamin A and C, suggesting the action of another caspase or a caspase-induced Ca^{2+} regulated serine protease (Lazebnik et al. 1995; Zhivotovsky et al. 1995).



Methods and interpretation

Antibodies against *PARP* and *lamin B* are available and can be used on methanol-fixed cryostat sections. Lamin B immunoreactivity cannot be demonstrated using formalin-fixed paraffin sections while PARP staining can be achieved on such sections following microwave pretreatment. PARP and lamin B have been termed ‘negative signals’ from cells in the later stages of the apoptosis cascade. Both antigens are known to be cleaved during apoptosis. Thus positive staining for both occurs in non-apoptotic cells while reduced or absent staining is found in cells that have already activated their execution caspases. The immunohistochemical approach on tissue sections gives particularly clear information for lamin B, which, prior to activation of execution caspases, is clearly identifiable as ring-like staining around the nuclear envelope; following activation of execution caspases lamin B is not detectable. The annular nature of nuclear staining can be readily discriminated from typical non-specific, diffuse staining artifacts found in many immunohistochemical reactions. The data obtained with PARP-antibodies are less easy to interpret as immunoreactivity (before activation of execution caspases) is diffuse and not always very conspicuous; following activation of the execution caspases nuclear immunoreactivity becomes even weaker. Because of this limitation, we suggest lamin B-immunoreactivity is currently the most reliable method to assess the activity of execution caspases on tissue sections. Both groups of antibodies can however be applied to intact cells in vitro (Fig. 10c, lamin B).

Fig. 10a–c Direct and indirect demonstration of activation of caspase 3 in isolated villous trophoblast in vitro. **a** Nomarski-interference contrast of isolated mononuclear fragments of syncytiotrophoblast (small structures) and trophoblast cells (larger cell bodies with large nuclei, *arrows*). **b** The enzyme histochemical proof for caspase 3 using PhiPhiLux in the identical field reveals activation of caspase 3 (fluorescence) only in at least two of the syncytial fragments rather than in the trophoblast cells. **c** The same trophoblast preparation: an antibody directed against lamin B only stains the larger nuclei of trophoblast cells (*arrows*); due to caspase 6-action this antigen can no longer be detected in the fragments of the syncytiotrophoblast. ×800

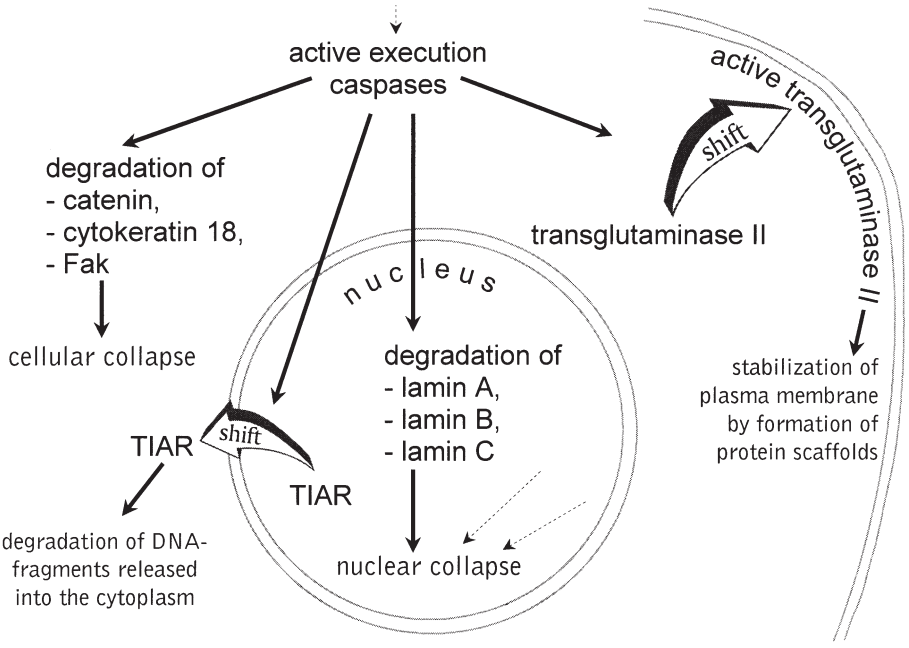


Fig. 11 Effects of active execution caspases on cleavage and intracellular shifts of proteins. The degradation of proteins involved in cytoskeletal functions (cytoplasm: catenin; cytokeratin 18, focal adhesion kinase, Fak; nucleus: lamins A, B, C) is responsible for structurally typical signs of apoptosis such as shrinkage and collapse of cell and nucleus. The mechanisms and consequences of protein shifts (TIAR: from the nucleus into the cytoplasm; transglutaminase II: from a diffuse cytoplasmic distribution to a strictly subplasmalemmal localization) are not yet fully understood but are thought to be useful marker events for the execution stages

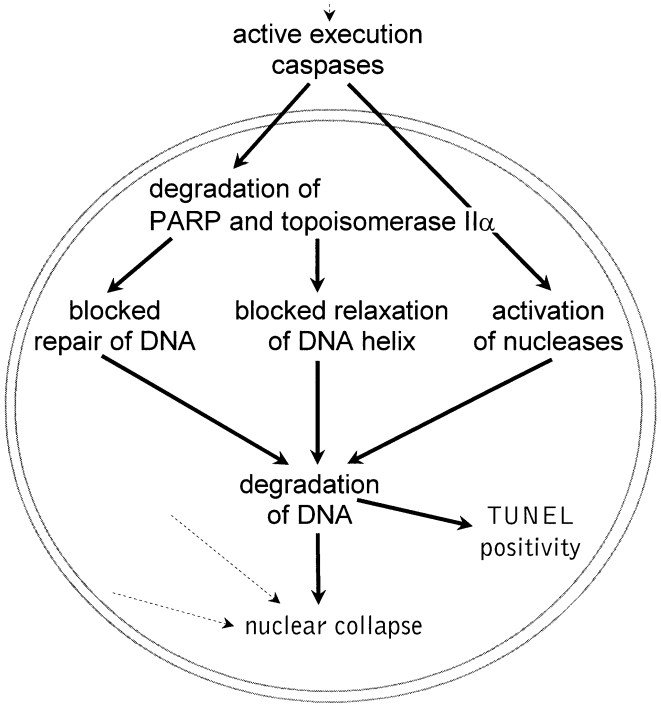


Fig. 12 Effects of active execution caspases on DNA degradation. Further targets of active execution caspases comprise proteins involved in DNA repair and maintenance such as PARP and topoisomerase IIα. Their inactivation in combination with caspase-dependent activation of endonucleases leads to single strand breaks of the DNA resulting in positivity of the TUNEL test

Cleavage of cytokeratin 18

Cytokeratin 18 is an intermediate filament protein cleaved by execution caspases (caspases 3, 6, 7; Caulin et al. 1997; Fig. 11). Conservation of a specific cleavage site within the cytokeratin 18 molecule and other intermediate filament proteins suggests that their processing may be a common phenomenon of caspase cleavage during apoptosis.

Methods and interpretation

Very recently a new antibody has been generated (Table 1) that, according to the manufacturer's protocol, binds to a caspase-cleaved formalin-resistant epitope of *cytokeratin 18*. This antibody can be used in paraffin sections for the immunohistochemical detection and localization of cleaved cytokeratin 18 during apoptosis. This is because the antibody recognizes a specific caspase cleavage site within cleaved cytokeratin 18 that is not present in native cytokeratin 18 of healthy cells (Leers et al. 1999). Cytoplasmic localization of this antibody points to activation of execution caspases and thus indicates that the cell has entered the early irreversible stages of apoptosis.

Intracellular shift of proteins caused by execution caspases

Directly following activation of the execution caspases, translocation of proteins from the nucleus to the cytoplasm, or within the cytoplasm, has been described (Fig. 11). The mechanistic links between caspase activation and commencement of these events awaits elucidation. Nevertheless the shift of these proteins provides a useful opportunity to employ immunohistochemistry for the identification of advanced, but not terminal, stages of apoptosis.

TIAR and transglutaminase II

One of these events is the shift of TIAR (T-cell restricted intracellular antigen related protein) from the nucleus to

the cytoplasm. Normally, TIAR is confined to the nucleus. However following activation of the execution caspases it is progressively released from the nucleus into the cytoplasm (Taupin et al. 1995). TIAR is a RNA-binding protein that cleaves DNA in vitro (Kawakami et al. 1992). Cytoplasmic shift of TIAR may be involved in binding of RNA clusters and/or destruction of DNA fragments that accumulate in the cytoplasm during apoptosis (Susin et al. 1997; Biggiogera et al. 1998).

In healthy, non-apoptotic, cells transglutaminase II (tissue transglutaminase) is evenly distributed throughout the cytoplasm. During later stages of apoptosis this enzyme is activated and shifts to a location beneath the plasma membrane. There it forms crosslinks between cytoplasmic proteins, resulting in the formation of extensive sub-plasmalemmal protein scaffolds (Cummings 1996; Fesus et al. 1996). These scaffolds are thought to prevent the release of cytoplasmic contents from the apoptotic cell into the interstitium, which might otherwise occur due to apoptosis-induced permeability of the plasma membrane in the later stages of the cascade (Piredda et al. 1997). By contrast with necrotic cell death, the release of potentially inflammatory/antigenic molecules during apoptosis is prevented.

Methods and interpretation

Antibodies suitable for immunohistochemical use are available for both antigens. They can be applied on formalin-fixed paraffin sections without the use of any pretreatment. Interpretation of *TIAR*-immunoreactivity may be problematic since even in seemingly non-apoptotic tissues TIAR-localization is not always restricted to the nucleus; rather it can also appear in a perinuclear position. It is unclear whether the perinuclear position of TIAR is an indication of early progression of the apoptosis cascade. It is therefore difficult to distinguish between non-apoptotic or early apoptotic cells when they have a small amount of cytoplasm surrounding the nucleus.

Such problems do not arise with *transglutaminase II*. Even though it is impossible to distinguish between the inactive proform and the active form by means of immunohistochemistry, meaningful data can be obtained because the diffuse cytoplasmic reactivity of the proform contrasts sharply with the strictly subplasmalemmal localization of the active enzyme. This point holds true for the application to tissue sections (Fig. 9c) as well as to intact cells in vitro. The only technical problem we have encountered is that optical refraction artifacts may occur along the plasmalemma when using interference contrast microscopy; these have the potential to be misinterpreted as evidence of a shift in transglutaminase II in non-apoptotic cells.

DNA fragmentation

In addition to the above events, active execution caspases will activate endonucleases including DFF (DNA fragmentation factor; Liu et al. 1997). These are responsible for a highly characteristic pattern of DNA fragmentation, producing a typical laddering of DNA fragments in an agarose gel. Visualization of endonuclease activity in tissue sections is best achieved using the TUNEL test (Fig. 12).

Methods and interpretation

In the *TUNEL* assay (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) the enzyme terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA single strand breaks generated principally by apoptosis signals. TdT catalyzes the addition of deoxynucleotides that, in the TUNEL kit are labeled with a visualization agent such as biotin. Subsequent addition and specific binding of streptavidin to biotin with further development by e.g., DAB results in an insoluble coloured substrate at the sites of DNA fragmentation (Fig. 13a).

The sensitivity of the assay is critical since single strand breaks of the DNA are found both during mitosis and throughout the whole life of a cell. Therefore a low level of DNA breakage exists in all cells and false positive results can easily be obtained, e.g., with excessive pretreatment with proteinase K, or excess TdT (for

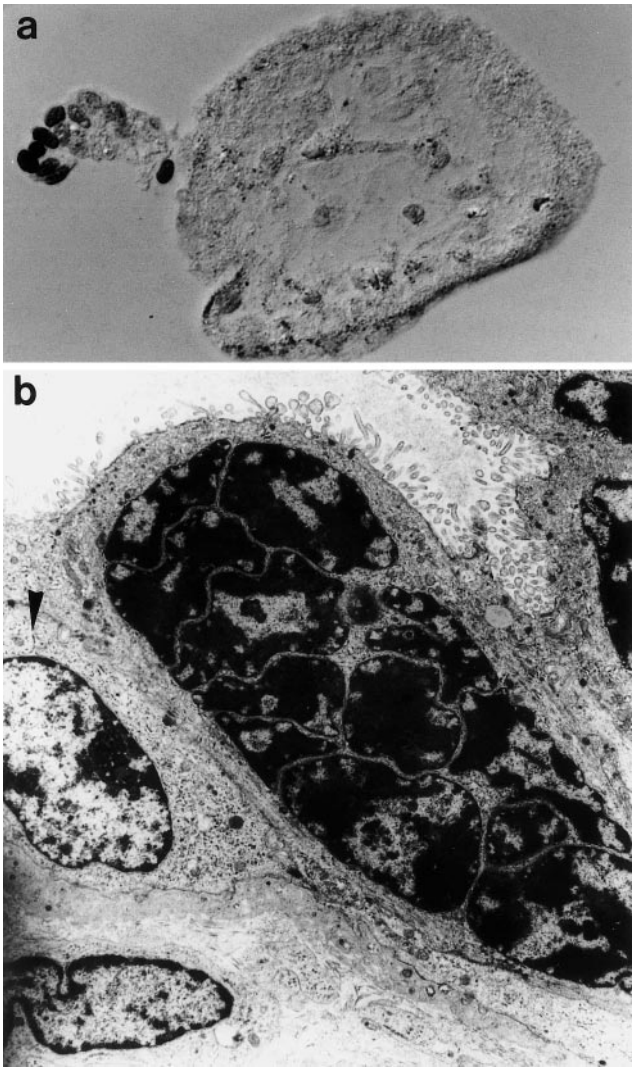


Fig. 13 **a** The breakdown of the DNA, as a very late event in the apoptosis cascade, can be visualized by the TUNEL test. TUNEL-positivity in the syncytiotrophoblast typically prevails in syncytial sprouts, which represent the sites of extrusion of apoptotic nuclei. ×800. **b** Also on the ultrastructural level the accumulation of aged nuclei with typically condensed chromatin is restricted to syncytial sprouts and knots. The underlying trophoblast cell (*arrowhead*) reveals normal nuclear morphology. ×5000

critical evaluations see Yasuda et al. 1995; Labat-Moleur et al. 1998). However, if performed accurately, the ensuing colour reaction is much easier to interpret for the non-morphologist than are more subtle structural alterations of the nucleus (see below). In our experience, the technique can be applied to both cryostat and formalin-fixed paraffin sections. We recommend selecting a TUNEL kit and adjusting it for every new tissue by careful comparison with nuclear morphology. For example TUNEL-test reactivity should be adjusted in pilot experiments so that specific staining occurs in nuclei with the typical annular chromatin condensation or in classic apoptotic bodies (see below).

Recently, a new antibody has been developed with the intention of using it as a tool for the detection of drug-induced DNA breaks (Frankfurt 1987). This monoclonal antibody can be used for the detection of single-stranded DNA and is suitable for use with formalin-fixed paraffin sections (Table 1).

Morphological alterations during apoptosis

Structural evidence of apoptosis becomes apparent during the later stages of the cascade since they reflect an extensive series of biochemical and enzymatic activities described in the previous sections. However some structural evidence of apoptosis is present during the early stages of apoptosis, generally prior to irreversible activation of the execution caspases. Such features include: loss of surface microvilli, formation of cell surface blebs and in some tissues (e.g., villous trophoblast, skeletal myoblasts, osteoclasts), the process of syncytial fusion. These structural alterations, viewed in isolation, cannot be taken as conclusive evidence of apoptosis.

Irreversible apoptosis is characterized by more obvious structural correlates. These indicate progression of the cascade beyond activation of execution caspases and include condensation of chromatin and alterations of nuclear shape. Chromatin condensation usually commences with a diffuse increase in nuclear density, followed by formation of spots of maximal density beneath the nuclear envelope. The final and highly characteristic structural change is one of annular chromatin condensation. These events are accompanied by a parallel loss of nuclear volume. In most, but not in all tissues (e.g., trophoblast) these events are typically followed by fragmentation of the cell and its nucleus, resulting in smaller apoptotic bodies. Macrophages eliminate the latter without induction of an inflammatory reaction.

Methods and interpretation

Most of the above structural alterations can be visualized by light microscopy in paraffin-embedded tissue sections (4–5-µm-thick). However, much thinner sections (typically 1 µm) can be cut following embedding in a plastic resin and these provide a much superior level of optical resolution of *nuclear morphology*. Finer analysis in particular of the intra-nuclear changes requires transmission electron microscopy (Fig. 13b). From the previous discussion it is important to emphasize that amongst the spectrum of structural alterations only those involving nuclear changes are diagnostic of apoptosis.

Conclusions

The cascade of events occurring during the apoptosis process is in many aspects still a mystery. And the nearly explosive increase of data available makes it unlikely that this confusion will become less in the near future. Even in recent reviews the question is posed whether even a basic model of the apoptosis cascade with a sequential activation of caspases can be valid for every cell, and whether the data obtained in vitro really reflect the in vivo situation (Villa et al. 1997). Rather, there is a continuously growing body of evidence that different cells have developed quite different modes to reach the final goal of apoptotic death. Accordingly, the cascade described above and the methodological considerations derived from it, cannot be applicable to every cellular system.

Recent findings have shown that the molecular machinery for apoptosis is not only required for programmed death of a cell but can also be used to reach certain steps of differentiation (Huppertz et al. 1998; Ishizaki et al. 1998; Morioka et al. 1998). One may even postulate that many stages of differentiation are in fact early stages of apoptosis (e.g., syncytial fusion); or, the earlier the stages of apoptosis we deal with, the more closely related to differentiation they are. Activation of caspases, cleavage of lamin B and generation of single strand breaks inside the DNA (TUNEL-positivity) are examples of events described not only for apoptosis but rather for differentiation (Ishizaki et al. 1998; Morioka et al. 1998). Leaving the proliferative stage and the cell cycle seems to be the signal for both ‘cascades’ – differentiation and apoptosis. In general, it becomes more and more difficult to define the differences between these events.

The trophoblast of the human placental villi provides a nice example of how intensely differentiation and apoptosis can be intertwined with each other (Huppertz et al. 1998):

Already the proliferating stem cells (villous cytotrophoblast, Langhans’ cells) start the apoptosis cascade, since very early steps of this cascade are needed for syncytial fusion. The programmed cell death will not continue in the stem cells as syncytial fusion leads to the formation of the highly differentiated syncytial tissue, the syncytiotrophoblast. Here, progression of apoptosis can only be inhibited for a certain time-span by continuous transfer of inhibitory members of the bcl-2 family (bcl-2 and mcl-1 protein and RNA) by syncytial incorporation of additional stem cells. Continuous syncytial fusion, initiated by early apoptosis in the cytotrophoblast, retards progression of apoptosis in the resulting syncytiotrophoblast; in contrast, stopping syncytial fusion leads within days to apoptotic death of the syncytiotrophoblast.

It is not the intention of this review to explain apoptosis. Rather, we want to present a spectrum of morphological tools for its study. Moreover, we want to highlight that simple analysis of the endstages of apoptotic death by transmission electron microscopy and/or TUNEL re-

activity cannot be sufficient to analyze the role of apoptosis in any tissue. Fortunately, today enough handy methods for visualization of the various stages of the apoptosis cascade are available.

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