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**Artikelen**

# Apoptosis - the genetically controlled physiological cell death: biochemistry and measurement

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There are two distinct mechanisms by which cells may die: an accidental cell death or necrosis, induced by a lethal physical, chemical or biological injury, and a programmed cell death (PCD), designated apoptosis (1,2). Apoptosis is an active bio-energy saving cell-elimination mechanism by which aged, unwanted or sublethal damaged cells are abolished and their contents with precious caloric value are utilised again by macrophages or by phagocytosing adjacent cells (1,2). During necrosis the cell membrane looses its selective permeability and ion-pumping capacity as result of a direct membrane damage. This leads almost instantaneously to swelling of the cell and its organelles, including the mitochondria, and leaking of the cellular contents into the extracellular space. Activation of enzymes such as hydrolases, phospholipases, proteases, RNases, and DNases results in further degradation of membranes, proteins, RNA and DNA, which accelerates the cellular and nuclear disintegration. Necrosis occurs in whole fields of damaged cells, where the leaked cellular debris elicits an inflammatory reaction in the adjacent viable tissues. During apoptosis a different and specific pattern of cell abolition takes place. The earliest changes include the loss of cell junctions and specialised membrane structures such as microvilli. The integrity of the cell membrane and of the mitochondria remains initially intact, the cytoplasma condenses and the nucleus coalesces into several large masses, which then break up into fragments. The endoplasmatic reticulum transforms into vesicles that fuse with the cytoplasmic membrane. These processes result in contraction of the cytoplasmic volume. The cell adopts a convoluted outline and subsequently the cell breaks up into small vesicles, so called apoptotic bodies. These vesicles enclose fractional parts of the cellular contents and several apparently intact organelles. The apoptotic bodies end up in the extracellular space, where they are phagocytosed by nearby cells and macrophages. Because apoptosis occurs in dispersed cells, the whole process takes only a few hours and the cell remnants do not elicit any inflammatory reaction, apoptotic cell death has long been overlooked. The various morphological differences between apoptosis (programmed cell death) and necrosis (accidental cell death) are summarised in table 1 (1,2).

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**Nonstandard abbreviations**

bcl-2: cell death regulator proto-oncogene; bax, bcl-XL, bcl-XS: members of the bcl-2 gene family; c-myc: cell-cyclus regulator oncogene; CM: ceramide; DAG: diacylglycerol; Fas/Apo-1: membrane receptor of cell death, a member of the TNF receptor family; Fas-L: Fas ligand, a member of the TNF family; ICE: interleukin-1ß converting enzyme; IGF-1: insulin-like growth factor-1; NGF: nerve growth factor; NGF-R: nerve growth factor receptor; PC: phosphatidylcholine; PCD: programmed cell death; PDGF: platelet-derived growth factor; PI: propidium iodide; PKC: protein kinase-C; PS: phosphatidylserine; P2Z: purinergic receptor type 2Z; p-53: tumor supressor gene; SM: sphingomyelin; TNF: tumor necrosis factor; TNF-R: tumor necrosis factor receptor; tTG: tissue transglutaminase; Yama: ICE related gene.

## Biochemistry of apoptosis: the cell death machinary

### Initiating Phase

The apoptotic pathway (figure 1) may become activated either by external signals that trigger receptors on the plasma membrane or by intracellular alterations, such as DNA damage, degenerative processes due to ageing or reactive oxygen species. The following receptors play a role in this process (figure 2):

*Fas/Apo-1 receptor.* A surface structure, implicated in triggering the process of apoptosis is Fas or APO-1 (figure 2). The APO-1 receptor was discovered as a cell surface molecule that could mediate apoptotic cell death in transformed human lymphocytes after exposition to a murine monoclonal antibody (3,4). Molecular cloning of Fas/APO-1 cDNA revealed that these two genes are identical (5,6). It has homology of about 70 amino acids to the receptors for tumour necrosis factor (TNF-R), nerve growth factor (NGF-R) and a variety of immune cell receptors CD27, CD30, CD40 (7). The structure of the receptors indicates that these molecules are “type-I” membrane proteins. An area of 65 amino acids has been defined as the “death domain” because it is responsible for promoting the death signal (7). In 1994 a cytokine has been described that binds to the Fas receptor. This

**Table 1.** Morphological and biochemical differences between accidental (necrosis) and programmed (apoptosis) cell death

| | Necrosis | Apoptosis |
|---|---|---|
| Origin | anoxia, starvation | lack of growth factor |
| | physical, chemical injury | hormonal influence<br>sublethal damage |
| Occult phase | none | minutes to hours |
| First manifestation | swelling | shrinking, convolution |
| Nuclear changes | granulation, karyolysis | initially intact, condensation |
| Membrane integrity | early failure | initially intact |
| Surface morphology | blebbing, lysis | smoothing, protrusions |
| Cytoskeletal changes | leakage of cellular contents | formation apoptotic bodies |
| Organelles | swollen, leaky | initially intact |
| Protein synthesis | not affected by inhibitors of protein synthesis | process affected by inhibitors of protein synthesis |
| Cytoplasmatic changes | rupture of lysosomes | endonuclease activity ↑ |
| | release of content | transglutaminase ↑<br>p53 ↑, bcl-2 ↓, c-myc ↑ |
| DNA degradation | diffuse degradation<br>→ DNA-smear | internucleosome cleavage<br>→ DNA-laddering |
| Cells affected | groups of contiguous cells, tissue areas | individual cells<br>scattered cells |
| Cell elimination | inflammatory response in adjacent tissues | engulfment by macrophages endothelial a.o. cells |

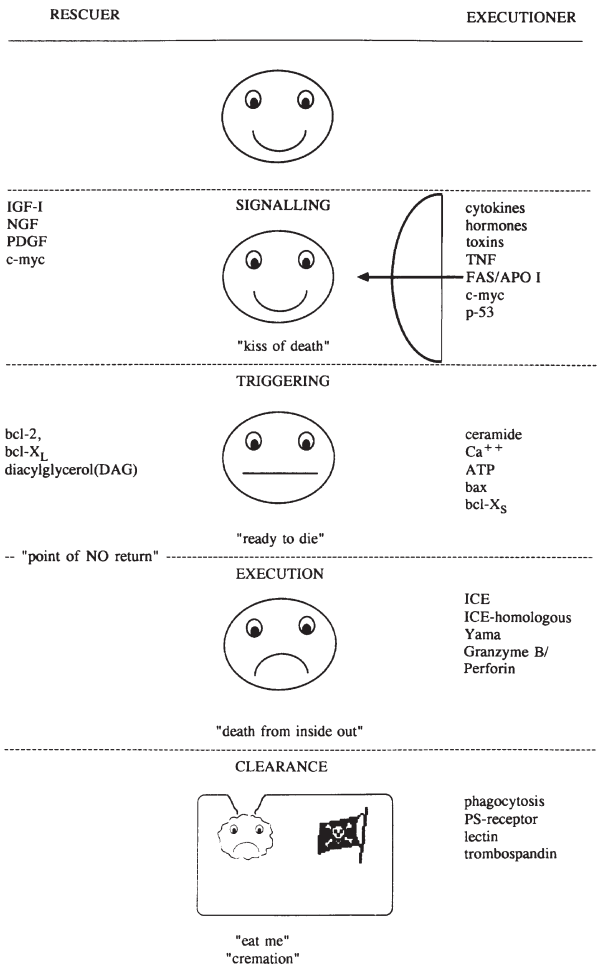


**Figure 1.** Schematic illustration of the molecular pathway of apoptosis.

ligand, indicated FasL, is believed to be a physiological inducer of apoptosis (8). The purified FasL appeared to be a protein with a molecular weight of 40 kDa, without signalling sequence at the NH2-terminus, suggesting that it is a "type-II" membrane protein with a COOH-terminal outside the plasma membrane. FasL belongs to the tumour necrosis factor (TNF) family.

*Purinergic receptors*. Another important surface structure, implicated in the starting of the apoptotic machinery in certain cells is the activation of a subclass of purinergic receptors: the purinoreceptor P2Z, which is sensitive to ATP (9,10). P2Z-receptor represents a ligand-gated ion channel, allowing ion-fluxes upon ligation with extracellular ATP (figure 2).

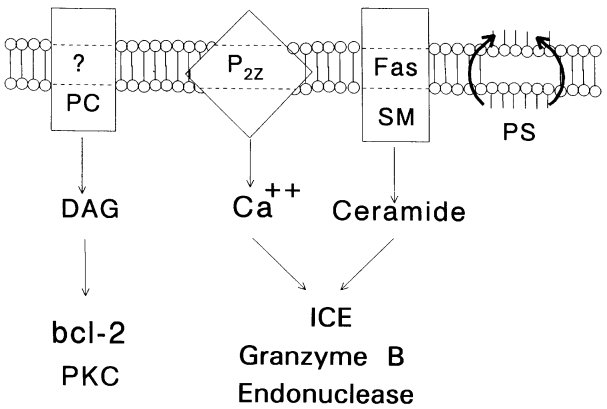


**Figure 2.** Schematic illustration of the membrane alterations during the initiating phase of apoptosis.

*Plasmatic receptors*. The cytoplasma contains receptors for growth factors and cytokines, withdrawal from a cell of chemical signals, known as survival factors may set apoptosis in motion (7). This mechanism assures that cells which have become deranged are eliminated. In classical endocrine target tissue such as mammary gland, the endometrium, the prostate, the cells undergo apoptosis when deprived of the appropriate target hormone (2). Comparable mechanisms occur in the hematopoietic tissue and in the nervous system, where the continuous presence of colony stimulating factors or nerve growth factor is required to suppress apoptotic cell death (2).

**Decision Phase**

The apoptosis inducing triggers from outside the cell are transduced to the cytosol resulting in a cascade of biochemical events that eventually may lead to apoptotic cell death. The whole process is controlled by positive (executioner) and negative (rescuer) regulatory elements (figure 1).

*Membrane alterations*. As a result of the signalling, that jeopardises the cell survival, changes occur in the plasma membrane, including alterations in the phospholipid architecture. One of the changes includes the appearance of phosphatidylserine (PS) in the outer leaflet of the plasma membrane. In living cells this phospholipid species is predominantly localised in the plasma membrane leaflet facing the cytosol (11,12). This redistribution is of important significance during the apoptotic process, because it occurs quite initially, while the plasma membrane as such, remains at first intact (13).

*Phospholipid seconder messengers*. The extracellular triggers for apoptosis, such as Fas-ligation, TNF, NGF, IL-2, glucocorticoids, sublethal chemical or physical injury (cytotoxic drugs, ionising irradiation) have been shown to induce shifts in $Ca^{++}$ compartments and to activate intracellular lipases and lipid-modifying enzymes, which results in formation of several phospholipid second messengers: ceramide (CM), diacylglycerol (DAG). Ceramide is formed from sphingomyelin by sphingomyelinase, DAG by enzymatic hydrolysis of phosphatidylcholine (14,15). DAG activates protein kinase-C (PKC) isoenzymes. Increasing intracellular levels of CM induces apoptosis in various cell lines and CM induced apoptosis is inhibited by the simultaneous presence or addition of DAG. The opposing effects of CM and DAG on the occurrence of apoptosis suggest that the decision of cells to live or die may be the result of two distinct pathways.

*Bcl-2 gene family.* The decision "to live or to die" is very much dependent from the expression of several distinct proto-oncogens, belonging to the *bcl-2 gene* family. These genes play a key role in the regulation of the apoptotic process (16,17). Bcl-2 represents a unique class of proto-oncogenes by its ability to block apoptosis without affecting cell proliferation.

**Execution Phase**

When the triggers have activated the membrane receptors and the membrane changes have resulted in $Ca^{++}$-ion influxes and production of second messengers then the effector elements become activated, which makes the process irreversible (18). The apoptotic machinery has passed the "point of no return", and the cell is primed for apoptosis ("ready to die"). The cell enters the phase of execution of the process. The effector elements ("executioners") are enzymes, facilitating degradation of intracellular structures and macromolecules, crosslinking proteins and preparing the cell for rapid phagocytosis (18). The final decision of apoptosis to occur may have a common pathway, that involves the activation of several enzymes:

*Proteases*. Interleukin-1ß converting enzyme (ICE) and ICE homologues (7,19) are evolutionary highly conserved cytoplasmatic cysteine protease belonging to a growing family of structurally related proteases. ICE and ICE-like proteases share the functionality of cleaving polypeptides next to an aspartic acid residue. Within the cell these proteases occur as inactive precursors, which can be activated by cleavage either by its own activated form (autocatalysis) or by other activated members of the family. The only other mammalian enzyme, presently known, with the same unusual cleavage preference next to aspartic acid is granzyme-B, a protease present in granules of cytotoxic lymphocytes and NK cells (20). Granzyme B together with a pore-forming enzyme perforin are implicated in the function of the cytotoxic effector cells in the cell-mediated cytotoxicity resulting in apoptosis of the target cells (21).

*Endonucleases*. During the process of apoptosis endogenous endonucleases are activated, which cleave linker DNA between nucleosomes, split DNA into large fragments and cleave single-strand DNA-chains. The endonuclease responsible for this chromatin fragmentation was found to be $Ca^{++}/Mg^{++}$-dependent and to be inhibited by $Zn^{++}$ (22). It has been suggested that the endonuclease is constitutively present in an inactive form in all cells, to become activated after the apoptotic signaling.

*Transglutaminases*. Cross-linking of proteins in apoptotic cells is due to the activity of a specific intracellular transglutaminase, a $Ca^{++}$-dependent cytosolic enzyme (23). This enzyme is present in high concentrations in cells undergoing apoptosis (23). It has been suggested that the function of this enzyme provides a highly cross-linked protein scaffold in apoptotic cells, joining cytoplasmic and membrane proteins and so maintaining cellular integrity during the formation of the apoptotic bodies and limiting the leakage of intracellular components into the extracellular space ("death from inside out") (24).

**Clearance Phase**

The remnants of the cell or apoptotic bodies are eliminated by phagocytosing adjacent cells and

macrophages (figure 1). A fascinating aspect of the apoptotic process is the recognition of apoptotic bodies by the phagocytes without causing damage to neighboring cells or eliciting an inflammatory response (25). The recognition of apoptotic cells and the subsequent phagocytosis by nearby cells are mediated by surface changes on the apoptotic cells and appropriate receptors on the engulfing cells. There is convincing evidence in the literature that the PS flip-flop phenomenon may act as a membrane "flag" on apoptotic cells (12,13). Due to this architectural change of the cell membrane, the surface hydrophobicity and charge are both altered, and this can be recognised by macrophage receptors (12).

**Measurement of apoptosis**

There are a number of adequate techniques to obtain information about cell proliferation, such as measurement of mitotic indices, labelling indices, DNA profile, and of the presence of nuclear antigens expressed by proliferating cells. On the other hand, tools that provide information about cell death either are not specific for apoptosis or lack quantitative values. In fact, the very nature of apoptosis can explain the technical difficulty to measure this "death from inside out" process. As stated before, the duration of apoptosis is short, involves single cells with morphological changes only after the "point of no return", ending in phagocytosis without inflammation. Therefore, it is no wonder that we are still far from a reference technique to measure apoptosis in a sensitive, specific and quantitative way. We can only briefly review here the methods which have been described to measure the occurrence of apoptosis in tissue and in cell suspensions, and for more details we can only refer to a very recent "user's guide"(26).

**Techniques Based on Morphological Changes**

*Measurement of apoptotic indices with light microscopy.* Characterisation of apoptotic cells is based on their specific morphological features such as bud formation, chromatin condensation and appearance of apoptotic bodies containing remnants of cell organelles and nuclei. In tissue sections these cells are observed as individual events. The proportion of apoptotic cells in a population can be quantified by counting cells visualised by light microscopy and accordingly expressed as the apoptotic index, being defined as the number of microscopic features per 100 cells that can be recognised e.g. in malignant tumours, exhibiting the morphological characteristics of apoptosis (26).

*Electron microscopy.* Electron microscopy is the method of choice when making detailed examination of the structural changes within cells but hardly a method for routine scoring of apoptosis. Scanning electron microscopy allows detailed examination of the cell-surface changes, including membrane blebbing and loss of features, such as microvilli. Within the cell, ultrastructural changes such as chromatin condensation and the absence of alteration in cytoplasmic organisation, can be assessed by transmission electron microscopy (26).

*Changes in light scatter patterns measured by flow cytometry.* As stated before, the integrity of the cytoplasmic membrane is lost immediately during necrosis but remains largely intact during the early stage of apoptosis. Later, during the process of cell death, cytoskeletal changes occur which, in the case of apoptosis, result in the formation of apoptotic bodies. These phenomena can be exploited in the flow-cytometer by the measurement of changes that occur in the cell scatter pattern. Forward light scatter reflects the cell diameter, while right angle scatter is a measure of inner cellular structures. During the initial stages of apoptosis, the cell membrane remains intact but the cell shrinks, while during necrosis cell swelling occurs immediately as a result of early failure of the cell membrane. This means that during the initial phases of apoptosis, forward light scatter diminishes, while right angle scatter temporarily increases or remains stable. On the other hand, during necrosis, the forward light scatter increases and the right angle scatter diminishes immediately (27,28). Unfortunately these parameters can only be evaluated on native cells in suspension, i.e. cells which have not been exposed to mechanical manipulation.

**Techniques Based on DNA Fragmentation**

*Measurement of the endonuclease activity.* The theoretical goal of a nuclease assay specific for apoptosis would be to measure the endonucleolytic cleavage of the genomic DNA at the internucleosomal sites. This type of assays is the most common biochemical method used for the detection of apoptosis, all based on the same simple principle: Nuclease enzyme-activity is a measure of the amount of the degraded DNA substrate. As substrate can be used exogenous DNA, a relatively large nucleic acid substrate isolated from non-apoptotic tissue nuclei, or endogenous DNA, when the substrate is the chromatin of the apoptotic nuclei. In both cases the main flaws are the specificity and sensitivity, because these assays are neither specific for internucleosomal cleavage nor sufficiently sensitive to recognise the individual apoptotic cells in a heterogeneous cell population. Direct measurement of the endonuclease-induced endogenous DNA fragmentation in extracts of apoptotic cells, until recently thought to be the hallmark of apoptosis, is the most common method to quantified apoptosis.

It was believed that the linker regions between nucleosomes are the DNA targets for the apoptotic-endonuclease attack, resulting in fragments of 180-200 bp and multiples of this unit length. This type of cleavage can be assessed by the appearance of a ladder of bands on a conventional agarose electrophoresis gel (29). Unfortunately this type of assay is essentially qualitative and not sufficiently sensitive to detect spontaneous apoptosis, or needs large number of cells which precludes usage of this assay to study apoptosis in vivo.

*Measurement of DNA content by flow cytometry.* As a result of the activation of an endonuclease, apoptotic cells exhibit a reduced DNA stainability, independent of the type of DNA-specific fluorochrome applied (27,28). By using e.g. propidium iodide (PI) apoptotic cells show a low DNA stainability, below the normal G0/G1 region, resulting in a sub G0/G1 peak designated as A0 cells (30). There is circumstantial evidence that this reduced DNA stainability may be the consequence of progressive loss of DNA from nuclei due to the activation of endogenous endonuclease and subsequent leakage of the low-molecular weight DNA product prior to measurement. In contrast to apoptotic cells, necrotic cells do not exhibit immediate reduction in DNA stainability (27,28,30).

*Labelling of DNA strand breaks.* Activation of the apoptosis-associated endonuclease results in extensive DNA cleavage and thus generates a large number of DNA strand breaks. The presence of 3'hydroxyl-termini of the strand breaks can be detected by labelling with modified nucleotides (e.g. biotin-dUTP, digoxigenin-dUTP, fluorescein-dUTP) in a reaction catalysed by exogenous enzymes like: terminal desoxynucleotidyl transferase (TdT) (31,32) or DNA polymerase (33). Fluorochrome conjugated avidin or digoxigenin antibodies are used in a second step of the reaction to render individual cells suitable for detection. Commonly used technique for the detection of apoptosis are the **i**n **s**itu **n**ick (ISN) labelling technique or the **T**dT-mediated X-d**U**TP **n**ick **e**nd **l**abelling (TUNEL). Both techniques are applicable for conventional histological sections (34) and for cell-suspensions using flow cytometry (28,32).

**Techniques Based on Membrane Alterations**

*Measurement of dye exclusion.* As we described previously, during apoptosis the integrity of the cytoplasmic membrane and a number of its basic functions remain intact. One of these functions is the active transport. Accordingly, apoptotic cells exclude non-vital dyes such as Trypan Blue or PI while necrotic cells do not (27,30).

*Probing for phospholipid redistribution: Annexin V assay.* Fadok and co-workers were the first to show that cell surface exposure of PS occurs in nucleated cell types during apoptosis (13). The observations of Fadok prompted us to study the interaction of Annexin V with apoptotic cells. The rationale for this study came from the knowledge that Annexin V binds specifically to the cell surface in the presence of $Ca^{++}$-ions, when PS is present on the outer leaflet of the plasma membrane (11). This property confers Annexin V with the ability to discriminate between PS exposing and non-exposing cells (11).
In analogy Annexin V appears to be a potent discriminator between viable and apoptotic cells (35,36). Using Annexin V as a FITC conjugate in combination with the propidium iodide (see above) one can distinguish between viable, apoptotic and secondary necrotic cells. The state of art arising from using this technology indicates that PS exposure is a universal phenomenon of apoptosis occurring in most if not all cell types independent of the initiating trigger (12,37). Cell surface exposure of PS precedes nuclear changes (15) and occurs downstream of the bcl-2 checkpoint (12). Hence, cell surface exposure of PS is an early marker of apoptosis during the initiating phase.
Due to its high affinity for PS containing membranes the Annexin V assay is easy to perform. Cells of interest and Annexin V-FITC are mixed in the presence of calcium. Propidium iodide may be added to this mixture in order to stain specifically the cells which have compromised plasma membrane integrity. Annexin V-FITC will bind immediately to cells which have surface exposed PS. Hence, after having prepared the reaction mixture it can be analysed almost instantaneously requiring neither prolonged incubation periods nor washing steps. Analysis can be carried out using fluorescence microscopy or flow cytometry. In this manner, viable, apoptotic and necrotic cells can be recognised easily. Viable cells will contain neither stain. Cells in apoptosis with intact plasma membrane integrity are stained only by Annexin V-FITC, whereas cells in necrosis, the phase consecutive to apoptosis *in vitro*, contain both stains (36) (figure 3).

**Techniques Based on Cytoplasmic Changes**

*Changes in intracellular enzyme activity.* We described earlier that during the execution phase of apoptosis, intracellular enzymes are playing a key role in this "eat me" process. It offers the theoretical possibility to measure the late apoptotic phase based on enzyme-activity. It has been demonstrated that activation of tissue transglutaminase (tTG) is part of the apoptotic machinery (23). tTG is activated in the dying cells to form cross-linked protein polymers/envelopes which can be extracted from cells with a significant rate of physiological cell death (23). Measurement of tTG activity can be done based on the incorporation of radioactive putrescine into casein, and with a sensitive enzyme-linked immunosorbent assay. There are several antibody preparations raised against tTG which have been used to detect and localise the tTG protein in apoptotic cells by immunohistochemistry and by immunoelectron-microscopy. In addition, the detection and localisation of tTG mRNA by in situ hybridisation are also reported (23).

*Measurement of calcium flux.* Elevations of the cytosolic $Ca^{++}$ level are also a result of the cell death. Energy-dependent $Ca^{++}$ transport system maintain the cytosolic $Ca^{++}$ concentration at 100 nmol/l, at least four orders of magnitude below that found in the extracellular space under physiological conditions. Accordingly, the increase of the cytosolic $Ca^{++}$ concentration could be a sensitive but temporary indicator of apoptosis. Although a number of different techniques have been historically used to demonstrate

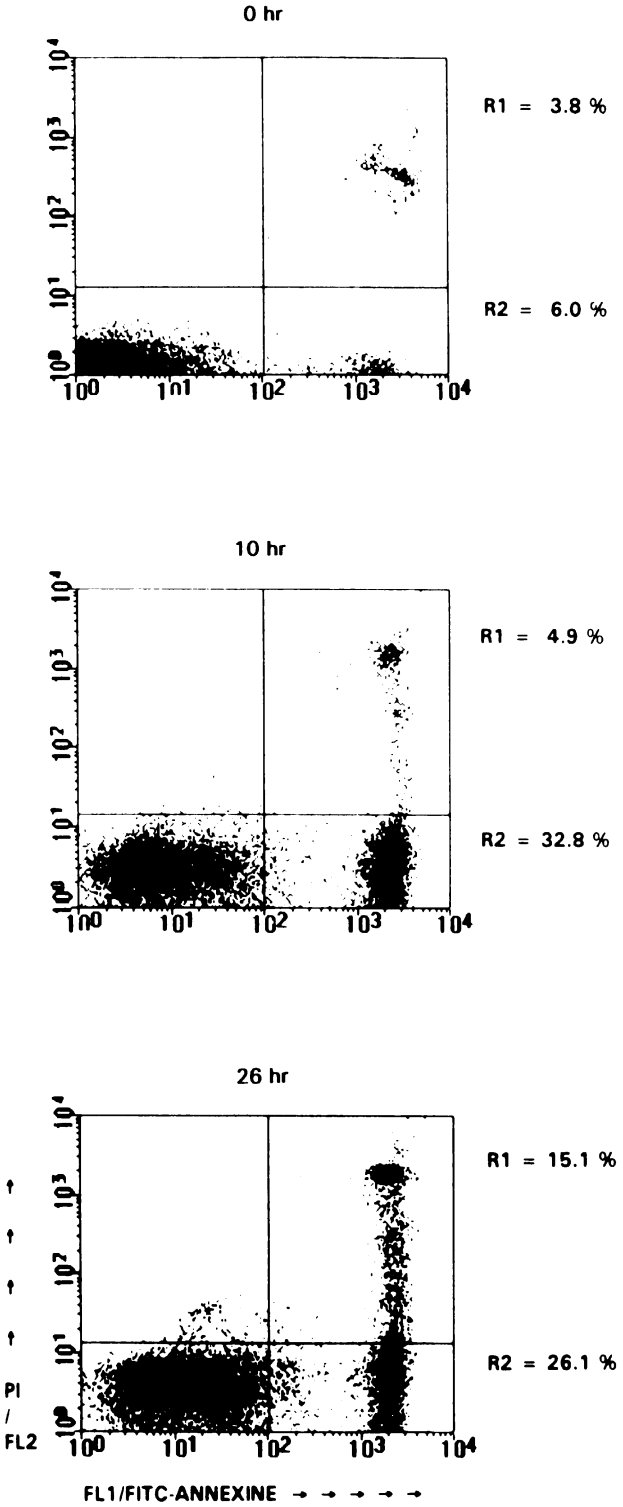


**Figure 3.** Probing for phospholipid redistribution: dot-plot of FITC-Annexin V (x-axis)/ Propidium Iodide (PI) (y-axis) two parameter flow cytometry of lymphocytes of patients with chronic lymphocytic leukemia at different time intervals during cultering. The lower left quadrants contain the viable cells negative for both the FITC-Annexin V binding and PI uptake. The lower right quadrants (R2) represent the apoptotic cells: positive for FITC-Annexin V binding and negative for PI uptake, demonstrating cytoplasmic membrane integrity. The upper right quadrants (R1) contain the necrotic cells positive for both FITC-Annexin V binding and for PI uptake.

**Table 2.** Diseases related to inappropriate apoptosis

| | |
|---|---|
| *Diseases related to excessive apoptosis* | |
| - Neurodegenerative disorders: | Alzheimer's disease<br>Parkinson's disease<br>amyotrophic lateral sclerosis<br>retinitis pigmentosa<br>spinal muscular atrophy<br>cerebellar degeneration<br>macular degeneration |
| - Myelodysplastic syndromes | |
| - Ischemic injury: | myocardial infarction<br>stroke |
| - AIDS | |
| - Acute hepatic atrophy | |
| - Tubular necrosis | |
| - Tumour lysis syndrome | |
| - Insulin-dependent diabetes | |
| - Toxin-induced liver disease | |
| - Osteoporosis | |
| *Diseases related to insufficient apoptosis* | |
| - Developmental abnormalities: | syndactylism<br>hypospadias<br>neural tube defect |
| - Proliferative diseases: | solid tumours<br>hormone-dependent tumours<br>follicular lymphomas |
| - Auto-immune diseases: | systemic lupus erythematosus<br>immune-mediated glomerulo-nephritis<br>autoimmune lymoproliferative syndrome |
| - Viral infections: | herpesviruses<br>adenoviruses<br>poxviruses |
| - Altered keratinisation: | eczema<br>psoriasis<br>hyperkeratosis |

$Ca^{++}$ fluxes, the use of $Ca^{++}$-selective fluorescent probes has replaced these other techniques as the strategy of choice for measuring changes in intracellular $Ca^{++}$ concentration in whole cells (38).

## Concluding remarks

The significance of apoptosis in clinical research and in clinical thinking has long been neglected. Biologists are now beginning to appreciate that the regulation of cell death is just as complex as the regulation of cell proliferation. The concept of programmed cell death offers new apprehension for a number of pathological syndromes and a better understanding for several clinical observations, which till now could not be explained by traditional cell-biological thinking. Such phenomena relate to congenital malformations, to the involution of tissues after hormonal deprivation, to ineffective hematopoiesis in myelodysplastic syndromes, to lymphocytolysis after

steroid therapy, to shrinkage of tumours after irradiation or cytotoxic therapy, to immuno-paralysis in AIDS, to discrepancy between cell proliferation and the actual tumour growth (39-41). The recent explosion in interest in apoptosis research is warranted given the substantial evidence that inappropriate apoptosis may contribute to the pathophysiology of several human diseases (table 2). These can be divided into disorders of enhanced apoptosis (e.g. neurodegenerative diseases) and those were aberrant (e.g. developmental abnormalities) or defective apoptosis (e.g. cancers) occurs (39-41). The concept of apoptosis challenges our conventional thoughts in immunology, carcinogenesis, cytotoxic therapy and radiobiology. Drug and therapy designs, which are directed to the modulation of the apoptotic process will offer new opportunities for treatment and control of diseases. If we find the measures to selectively manipulate the occurrence of apoptosis by suppression or stimulation, these would provide us with essentially new therapeutic strategies in a number of diseases. The concept of apoptosis as an "in vivo prognostic factor" is changing our point of view concerning anticancer drugs and offers new possibilities for cancer treatment.

In the near future the clinical laboratory has to offer techniques to measure tumour cell sensitivity to irradiation, to cytotoxic drugs and to combined treatments to provide the clinicians objective data for optimal drug choice. The measurement of the modulating effects of biological response modifiers, like cytokines, growth factors and hormones, upon the apoptotic efficacy of individual drugs offers the experimental conditions to test the advantageous or deleterious effects of drug combinations and provides the rational for development of novel treatment modalities. Cell death monitoring ex vivo offers the rational for optimal drug choice and individualisation of therapy.

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**Literature**

1. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26: 239-257.
2. Wyllie AH, Kerr JFR, Currie AR. Cell death: The significance of apoptosis. Int Rev Cytol 1980; 68: 251-300.
3. Trauth BC, Klas C, Peters AMJ, Matzku S, Möller P, Falk W, Debatin KM, Krammer PH. Monoclonal antibody-mediated tumour regression by induction of apoptosis. Science 1989; 245: 301-305.
4. Yonehara S, Ishii A, Yonehara M. A cell killing monoclonal antibody (anti-Fas) to a cell surface antigen co-down-regulated with the receptor of tumour necrosis factor. J Exp Med 1989; 169: 1747-1756.
5. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediated apoptosis. Cell 1991; 66: 233-243.
6. Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dhein J, Trauth BC, Ponstingl H, Krammer PH. Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumour necrosis factor / nerve growth factor receptor superfamily: sequence identity with the Fas antigen. J Biol Chem 1992; 267: 10709-10715.
7. Chinnaiyan AM, Dixit VM. The cell-death machine. Curr Biol 1996; 6: 555-562.
8. Valentine MA, Licciardi KA. Recue from anti-IgM-induced programmed cell death by B cell surface protein CD20 and CD40. Eur J Immunol 1992; 22: 3141-3148.
9. Di Virgilio F, Pizzo P, Zanovello P, Bronte V, Collavo D. Extracellular ATP as a possible mediator of cell-mediated cytotoxicity. Immunol Today 1990; 11: 274-277.
10. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. The cytolytic P2z receptor for extracellular ATP identified as a P2x receptor (P2X7). Science 1996; 272: 735-738.
11. Andree HAM, Reutelingsperger CPM, Hauptmann R, Hemker HC, Hermens WT, Willems GM. Binding of vascular anticoagulant a (VACa) to planar phospholipid bilayers. J Biol Chem 1990; 265: 4923-4928.
12. Martin SJ, Reutelingsperger CPM, McGahon AJ, Rader JA, Schie RCAA van, LaFace DM, Green DR. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. J Exp Med 1995; 182: 1545-1556.
13. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DI, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992; 148: 2207-2216.
14. Hannun YA, Obeid L. Ceramide: an intracellular signal for apoptosis. Trends Biochem Sci 1995; 20: 73-77.
15. Pushkareva M, Obeid LM, Hannum YA. Ceramide: an endogenous regulator of apoptosis and growth suppression. Immunol Today 1995; 16: 294-296.
16. Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes : Regulators of cell death. Blood 1992; 80: 879-886.
17. Yang E, Korsmeyer SJ. Molecular thantopsis: A discourse on the BCL2 family and cell death. Blood 1996; 88: 386-401.
18. Green DR, Martin SJ. The killer and the executioner: how apoptosis controls malignancy. Curr Opin Immunol 1995; 7: 694-703.
19. Patel T, Gores GJ, Kaufmann SH. The role of proteases during apoptosis. FASEB J 1996; 10: 587-597.
20. Irmler M, Hertig S, MacDonald HR, Sadoul R, Becherer D, Proudfoot A, Solari R, Tschopp J. Granzyme A is an interleukin 1ß-converting enzyme. J Exp Med 1995; 181: 1917-1922.
21. Smyth MJ, Trapani JA. Granzymes: exogenous proteinases that induce target cell apoptosis. Immunol Today 1995; 16: 202-206.
22. Cohen JJ, Duke RC. Apoptosis and programmed cell death in immunity. Ann Rev Immunol 1992; 10: 267-293.
23. Fesus L, Davies PJA, Piacentini M. Apotosis: Molecular mechanisms in programmed cell death. Eur J Cell Biol 1991; 56: 170-177.
24. Tinnemans MMFJ, Lenders MHJH, Velde GPM ten, Ramaekers FCS, Schutte. Alterations in cytoskeletol and nuclear matrix associated proteins during apoptosis. Eur J Cell Biol 1995; 68: 35-46.
25. Savill J, Fadok V, Henson P, Haslett C. Phagocyte recognition of cell undergoing apoptosis. Immunol Today 1993; 14: 131-136.
26. Cotter TG, Martin SJ. Techniques in Apoptosis. A User's Guide 1996; pp.1-331. London: Portland Press Ltd.
27. Darzynkiewicz Z, Bruno S, DelBino G, Gorczyca W, Hotz MA, Lassota P, Targanos F. Features of apoptotic cells measured by flow cytometry. Cytometry 1992; 13: 795-808.

28. Darzynkiewicz Z, Li X. Measurements of cell death by flow cytometry. In "Techniques in apoptosis. A user's guide"; 1996 edited by Cotter TG and Martin SJ, pp 71-106 London: Portland Press Ltd.
29. Wolfe JT, Pringle JH, Cohen GM. Assays for the measurement of DNA fragmentation during apoptosis. In "Techniques in apoptosis. A user's guide" 1996; edited by Cotter TG and Martin SJ. pp 51-70 London: Portland Press Ltd.
30. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 1991; 139: 271-279.
31. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 1992; 119: 493-501.
32. Gorczyca W, Bruno S, Darzynkiewicz RJ, Gong J, Darzynkiewicz Z. DNA strand breaks occurring during apoptosis: Their early in situ detection by the terminal deoxynucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. Int J Oncol 1992; 1: 639-648.
33. Meyaard L, Otto SA, Jonker RR, Mijnster MJ, Keet RPM, Miedema F. Programmed death of T cells in HIV-1 infection. Science 1992; 257: 217-219.
34. Wijsman JH, Jonker RR, Keijzer R, Velde CJH van de, Cornelisse CJ, Dierendonck JH van. A new method to detect apoptosis in paraffin sections: In situ end-labelling of fragmented DNA. J Histochem Cytochem 1993; 41: 7-12.
35. Koopman G, Reutelingsperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST, Oers MHJ van. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 1994; 84: 1415-1420.
36. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Meth 1995; 184: 39-51.
37. Engeland M van, Ramaekers FCS, Schutte B, Reutelingsperger CPM. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 1996; 24: 131-139.
38. McConkey DJ. Calcium flux measurement in cell death. In "Techniques in apoptosis. A user's guide" edited by Cotter TG and Martin SJ, pp 133-148, London: Portland Press Ltd.
39. Haanen C, Vermes I. Apoptosis and inflammation. Mediators Inflamm 1995; 4: 5-15.
40. Haanen C, Vermes, I. Apoptosis: Programmed cell death in fetal development. Eur J Obstet Gynecol Reprod Biol 1996; 63: 129-133.
41. Vermes I, Haanen C. Apoptosis and programmed cell death in health and disease. Adv Clin Chem 1994; 31: 177-246.

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**Summary**

*Apoptosis - the genetically controlled physiological cell death: biochemistry and measurement. Vermes I, Haanen C and Reutelingsperger CPM. Ned Tijdschr Klin Chem 1997; 22: 43-50.*

Apoptosis, or programmed cell death, is an orderly and genetically controlled form of cell death. In a morphological sense it differs from necrosis in that cellular shrinkage and chromatin condensation occurs, followed by fragmentation and distribution of nuclear and plasmatic components within membrane-bound vesicles (apoptotic bodies), which are cleared by phagocytosis without damage to adjacent tissue. The molecular pathway includes an Initiating phase, which starts after signalling by external triggers, such as ligation to distinct receptors or by endogenous mechanisms, related to ageing or to irreversible cellular or nuclear damage. The initiation phase is followed by the Decision phase. During this phase transduction occurs of the apoptotic signal to nuclear and cytoplasmic target enzymes, which includes activation of endonucleases and enzymatic alteration of the cytoskeleton. There are numerous proteins and lipid-derived moieties which modulate the apoptotic mechanisms in positive or negative directions. When the cell has arrived at a stage of no return than the Execution phase is started. The nuclear DNA is cleaved into multiples of 180-200 base pairs, the plasma membrane integrity and the mitochondria remain initially intact, the cell splits up into apoptotic bodies, small vesicles which enclose the nuclear and cellular remnants. Finally the Clearance phase is arrived, when the apoptotic bodies are phagocytosed by adjacent cells and macrophages. The apoptotic process can be measured based on its morphology (by light microscopy, electron microscopy or by flow cytometry), based on the DNA fragmentation (endonuclease activity, measurement of DNA content or labelling of DNA strand breaks), based on membrane alteration (dye exclusion, Annexin V probe) or based on cytoplasmic changes (intracellular enzyme activity, $Ca^{++}$ flux). Recent evidences suggest that alterations in apoptosis contribute to the pathogenesis of a number of human diseases including cancer, viral infections (AIDS), autoimmune disorders, neurodegenerative diseases, a.o.. Arteficial modification of the tendency to apoptosis may offer a novel treatment stategy for a number of these diseases. It is a challenge in pharmacological research to develop new drugs which affect the apoptotic process in positive or negative direction. Cell death monitoring *ex vivo* offers the rational for optimal drug choice and individualisation of various treatment regimen.

*Keywords: apoptosis; programmed cell death; molecular biology; measurement.*