
Review Article

Cytometry in Cell Necrobiology: Analysis of Apoptosis and Accidental Cell Death (Necrosis)

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The term cell necrobiology is introduced to comprise the life processes associated with morphological, biochemical, and molecular changes which predispose, precede, and accompany cell death, as well as the consequences and tissue response to cell death. Two alternative modes of cell death can be distinguished, apoptosis and accidental cell death, generally defined as necrosis. The wide interest in necrobiology in many disciplines stems from the realization that apoptosis, whether it occurs physiologically or as a manifestation of a pathological state, is an active mode of cell death and a subject of complex regulatory processes. A possibility exists, therefore, to interact with the regulatory machinery and thereby modulate the cell's propensity to die in response to intrinsic or exogenous signals. Flow cytometry appears to be the methodology of choice to study various aspects of necrobiology. It offers all the advantages of rapid, multiparameter analysis of large populations of individual cells to investigate the biological processes associated with cell death. Numerous methods have been developed to identify apoptotic and necrotic cells and are widely used in

various disciplines, in particular in oncology and immunology. The methods based on changes in cell morphology, plasma membrane structure and transport function, function of cell organelles, DNA stability to denaturation, and endonucleolytic DNA degradation are reviewed and their applicability in the research laboratory and in the clinical setting is discussed. Improper use of flow cytometry in analysis of cell death and in data interpretation also is discussed. The most severe errors are due to i) misclassification of nuclear fragments and individual apoptotic bodies as single apoptotic cells, ii) assumption that the apoptotic index represents the rate of cell death, and iii) failure to confirm by microscopy that the cells classified by flow cytometry as apoptotic or necrotic do indeed show morphology consistent with this classification. It is expected that flow cytometry will be the dominant methodology for necrobiology. Cytometry 27:1-20, 1997.

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Key terms: endonuclease; DNA cleavage; chromatin condensation; annexin V; light scatter

The subject of cell death has recently become a focus of interest for researchers from a variety of diverse fields, including cell and molecular biology, oncology, immunology, embryology, endocrinology, hematology, and neurology. The interest expands to a wide range of molecular, biochemical, and morphological changes which not only pertain to the actual act of cell death, but also predispose the cell to respond to an environmental or intrinsic signal by death, regulate the initial steps leading to irreversible commitment to death, and activate the postmortem cell disposal machinery. Because the scope of this field is so wide and involves many disciplines, we introduce the term *cell necrobiology* to comprise various modes of cell death; the biological changes which predispose, precede,

and accompany cell death; as well as the consequences and tissue response to cell death. The term combines *necros* ("death") and *bios* ("life"), which on first thought appears contradictory. However, in preparation for and during cell death, a complex cascade of biological pro-

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cesses, typical of cell life, takes place. These processes involve activation of many regulatory pathways, preservation and often modulation of transcriptional and translational activities, the function of cell organelles, activation of many diverse enzyme systems, modification of the cell plasma membrane structure and function, etc. A term that refers to the biology of cell death, thus, is not a contradiction. The term *cell necrology*, however, may apply to studies dealing with the subsequent postmortem changes, as will be discussed further.

CLASSIFICATION OF THE MODES OF CELL DEATH

Cell death is the process which culminates with cessation of biological activity. In contrast to cell quiescence or dormancy, which is also characterized by decreased rates of many biological activities, the changes which accompany death are irreversible. It is often difficult to define at which point a cell has passed the point of no return in the death process and which changes cannot be reversed. As an operational definition of cell death, independent of the techniques measuring it, one may accept the passage of the cell through such a point.

It has been generally accepted that apoptosis and necrosis are two distinct, mutually exclusive, modes of cell death (reviews: 2, 9,12,13,54,67,101,104,105). Apoptosis, frequently referred to as "programmed cell death," is an active and physiological mode of cell death, in which the cell itself designs and executes the program of its own demise and subsequent body disposal. A multistep mechanism regulates the cell's propensity to respond to various stimuli by apoptosis, whose complexity has only recently become apparent (74). The regulation system involves the presence of at least two distinct checkpoints, one controlled by the *bcl2/bax* family of proteins (47,65,81), another by the cysteine- (30,57,70) and possibly by the serine- (4,39,44,102) proteases. Through several oncogenes and tumor suppressor genes such as p53, this system interacts with the machinery regulating cell proliferation and DNA repair. Regulatory mechanisms associated with apoptosis are the subject of recent reviews (74,101). Several review articles discuss antitumor strategies based on modulation of the cell's propensity to undergo apoptosis, a subject of great interest in oncology in recent years (15,22,31,46,53).

A cell triggered to undergo apoptosis activates a cascade of molecular events which lead to its total disintegration. Because many of these changes are very characteristic and appeared to be unique to apoptosis, they have become markers used to identify this mode of cell death biochemically, by microscopy or cytometry (Figs. 1 and 2). One of the early events is cell dehydration. Loss of intracellular water leads to condensation of the cytoplasm followed by a change in cell shape and size: the originally round cells may become elongated and generally, are smaller. Another change, perhaps the most characteristic feature of apoptosis, is condensation of nuclear chromatin. The condensation starts at the nuclear periphery, and the condensed chromatin often takes on a concave shape resembling a

half-moon, horseshoe, or sickle. The condensed chromatin has an uniform, smooth appearance, with no evidence of any texture normally seen in the nucleus. DNA in condensed (pycnotic) chromatin exhibits hyperchromasia, staining strongly with fluorescent or light absorbing dyes. The nuclear envelope disintegrates, lamin proteins undergo proteolytic degradation, followed by nuclear fragmentation (karyorrhexis). Many nuclear fragments, which stain uniformly with DNA dyes and thereby resemble DNA droplets of different sizes, are scattered throughout the cytoplasm (Fig. 2). The nuclear fragments, together with constituents of the cytoplasm (including intact organelles), are then packaged and enveloped by fragments of the plasma membrane. These structures, called "apoptotic bodies," are then shed from the dying cell. When apoptosis occurs in vivo apoptotic bodies are phagocytized by neighboring cells, including those of epithelial or fibroblast origin (i.e., not necessarily by "professional" macrophages), without triggering an inflammatory reaction in the tissue (12,54,67,104,105).

Activation of endonuclease(s) which preferentially cleave DNA at the internucleosomal sections is another characteristic event of apoptosis (2,12,105). The products of DNA degradation are nucleosomal and oligonucleosomal DNA sections which generate a characteristic "ladder" pattern during agarose gel electrophoresis. As a result of the DNA in apoptotic cells being partially degraded, the fraction of low molecular weight DNA can be easily extracted (2,12,37). It should be pointed out, however, that in many cell types, DNA degradation does not proceed to nucleosomal sized fragments but rather results in 50–300-kb DNA fragments (72).

Another characteristic feature of apoptosis is the preservation, at least during the initial phase of cell death, of the structural integrity and most of the plasma membrane function. Also, cellular organelles, including mitochondria and lysosomes, remain preserved during apoptosis, although the mitochondrial transmembrane potential is markedly decreased (4a,17,44a,64). Other features of apoptosis include mobilization of intracellular ionized calcium (69), activation of transglutaminase which cross-links cytoplasmic proteins (80), loss of microtubules (26), loss of asymmetry of the phospholipids on the plasma membrane leading to exposure of phosphatidylserine on the outer surface (29), and other plasma membrane changes which precondition remnants of the apoptotic cell to become a target for phagocytizing cells. The duration of apoptosis may vary, but generally is short, even shorter than the duration of mitosis (54,104). Thus, under conditions of tissue homeostasis, when the rate of cell death is balanced by the rate of cell proliferation, the mitotic index may exceed the index of apoptosis.

While apoptosis is characterized by an active participation of the affected cell in its own demise, even to the point of triggering (in some cell systems) the *de novo* synthesis of the effectors of cell death, necrosis is a passive, catabolic, and degenerative process. Necrosis generally represents a cell's response to gross injury and

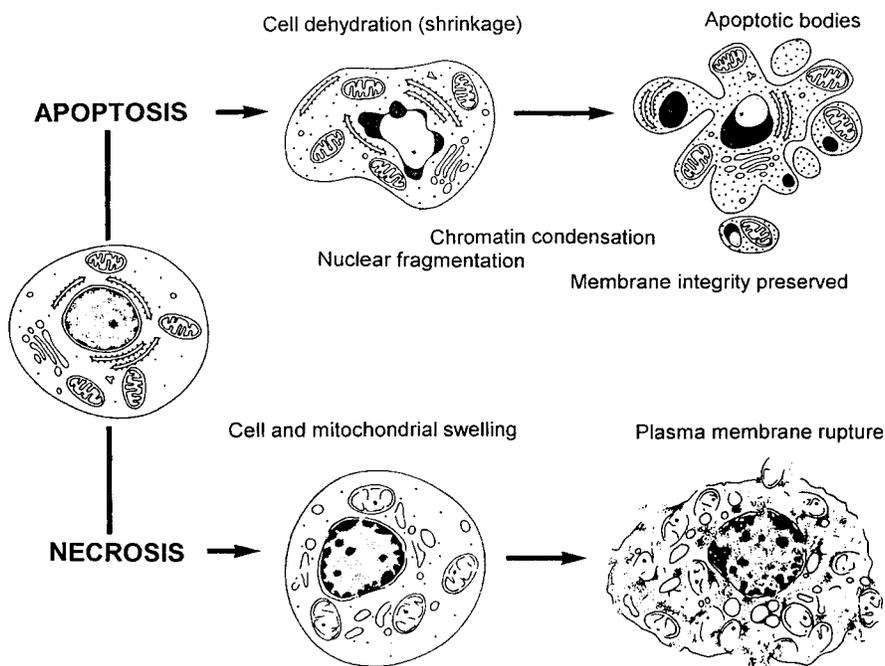


FIG. 1. Scheme illustrating morphological and biochemical changes during apoptosis and necrosis. One of the early changes during apoptosis is loss of intracellular water and increase in the concentration of ionized calcium in the cytoplasm. Chromatin condensation, followed by nuclear disintegration and formation of apoptotic bodies represent other typical features of apoptosis. The integrity of the plasma membrane is preserved to the late stages of apoptosis. Activation of endonuclease(s) results in selective degradation of DNA to 50–300-kb sections and is followed (in

many but not all cell systems) by internucleosomal DNA cleavage. Activation of Cys and Ser proteases results in selective degradation of some proteins such as poly(ADP)ribose polymerase and U1 small nuclear ribonucleoprotein. Swelling of mitochondria as well as swelling of the whole cell, combined with marginal chromatin condensation characterize the early stage of necrosis. Rupture of the plasma membrane occurs later and the content of cytoplasm is released from the cell. See text for further details.

can be induced by an overdose of cytotoxic agents. If apoptosis can be compared to cell suicide, necrosis is accidental death and is often referred to as “cell murder” (67). The early event of necrosis is mitochondrial swelling followed by rupture of the plasma membrane and release of cytoplasmic constituents, which include proteolytic enzymes (67,104). Nuclear chromatin shows patchy areas of condensation, and the nucleus undergoes slow dissolution (karyolysis). Necrosis triggers an inflammatory reaction in the tissue and often results in scar formation. DNA degradation is not so extensive during necrosis as in the case of apoptosis, and the products of degradation are heterogenous in size, failing to form discrete bands on electrophoretic gels.

It should be mentioned that cell death is not always accompanied by the classical features of either apoptosis or necrosis. Examples of cell death have been described in which the pattern of morphological and/or biochemical changes neither resembled typical apoptosis nor necrosis but often had features of both (1,5,10,11,34,77, 108). In some cases, the integrity of the plasma membrane was preserved, but DNA degradation was random, without evidence of internucleosomal cleavage. In other situations, DNA degradation was typical of apoptosis, but nuclear fragmentation and other features of apoptosis were not apparent. Generally, while most hematopoietic lineage

cell types are “primed” to apoptosis and their death has typical features of apoptosis, the death of epithelial type cells is more complex and sometimes difficult to classify. Furthermore, some drugs which cause apoptosis may additionally confuse the pattern of cell death due to the drug-induced secondary effects on the cell. For example, when apoptosis is triggered by drugs affecting cell structure and function, or by drugs affecting one or more pathways of the apoptotic cascade, particular features of apoptosis may not be apparent. Likewise, prolonged cell arrest in the cell cycle induced by some drugs leads to growth imbalance which may dramatically alter cell biochemistry and morphology (38).

A variety of names are assigned, not always correctly, to a particular mode of cell death. Cell death by suicide, active or physiological cell death, or shrinkage necrosis are frequently used as synonyms of apoptosis; the latter term is preferred by pathologists. The term *programmed cell death* also is frequently used as being synonymous with apoptosis. Such use, however, is erroneous since this term denotes a program of cell death in the sense of activation of a genetic clock for eliminating the cell, and has no relation to the mechanism of cell death. Actually, there are situations when apoptosis appears not to occur as the result of programmed cell death (e.g., 88).

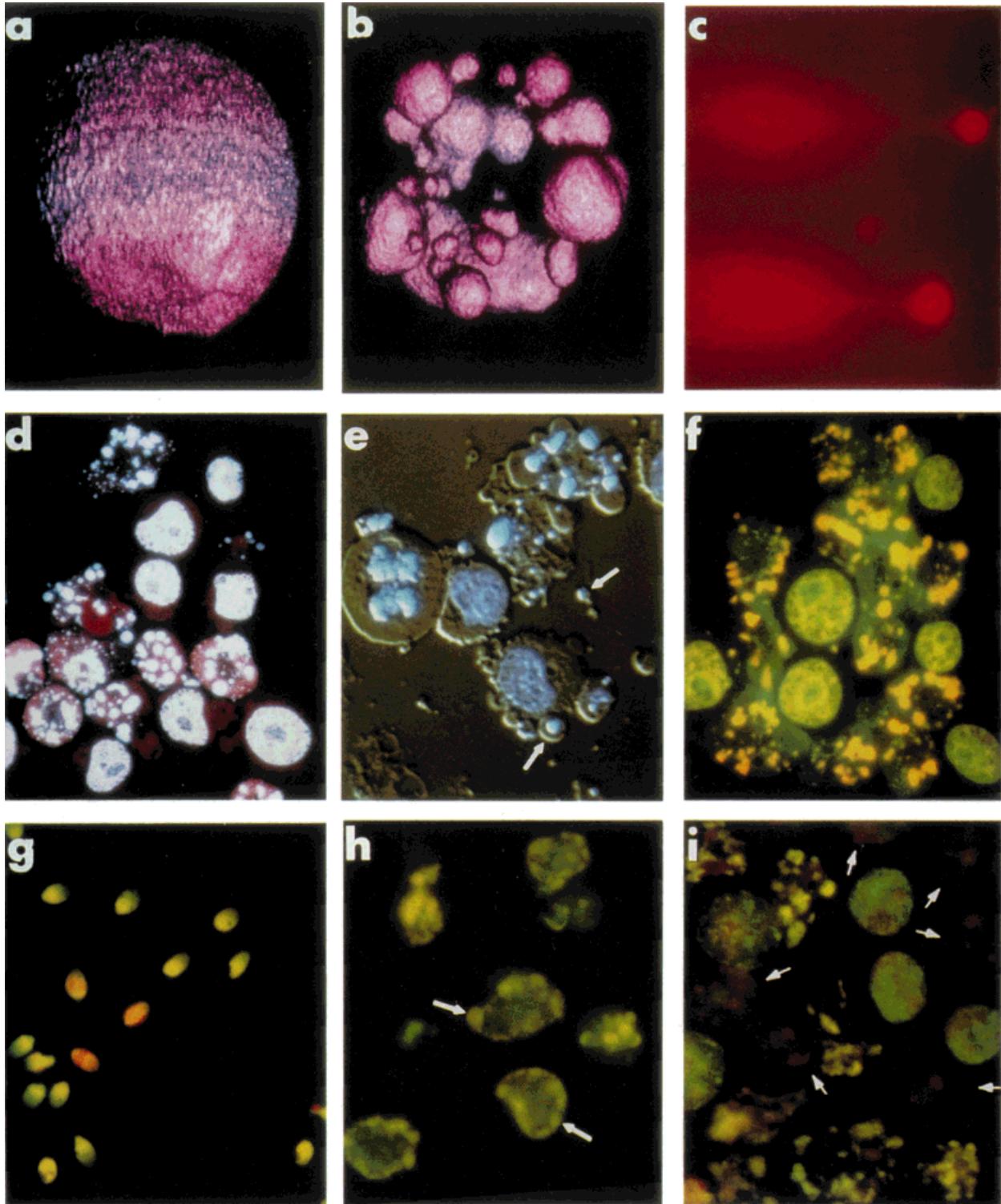


FIG. 2 (Legend on facing page).

Reproductive or mitotic cell death or mitotic catastrophe are terms used in the literature to describe cell death which occurs in the cell cycle subsequent to the one during which the cells was exposed to the toxic agent (6,90). The cell, while it may transiently become arrested in the cycle as a result of the damage, is able to divide. Its progeny cells, however, die when progressing through the subsequent cycle(s). It is likely that their death occurs during a transition through one of the checkpoints of the cell cycle. The cause of their death may be damage to genes which are essential for cell survival. The originally sublethal damage may be amplified and/or become a lethal lesion during the next round of DNA replication and mitosis. In addition, severe growth imbalance may occur when the cell is arrested in the cell cycle, but RNA and protein synthesis continue. The secondary changes in these cells, resulting from DNA damage and growth imbalance often cause an alteration in cell morphology and metabolism to such an extent that it is difficult to classify their mode of death. Such cells may show the features of both apoptosis and necrosis or have very atypical changes which lack the key morphological or biochemical features of apoptosis (5,90,99).

In contrast to reproductive or mitotic cell death, the term *interphase cell death* is used to describe the death of cells, following injury, which occurs prior to their division (90,96). This term does not define the mechanism of death. In many instances (e.g., induction of S phase cell death by the DNA topoisomerase I inhibitor camptothec-

in) interphase cell death occurs by apoptosis. In other instances, however (e.g., induction of cell death by ionizing radiation), the severity of the cell's injury, which can rapidly trigger cell death, may lead to cell necrosis.

Recent articles by Majno and Joris (67) and Levin (58) provide an exhaustive and stimulating overview of the complexity in defining the mode of cell death and in classification of different death mechanisms. Majno and Joris convincingly argue that cell death and necrosis are two quite different events (Fig. 3). Cell death, according to these authors, is the point of no return; past this point are the postmortem changes, which these authors define as necrotic changes. In the case of induction of liver cell death by ischemia, for example, the irreversible point is at approximately 150 min after onset of oxygen deprivation (67). At that time, however, no significant morphological changes can be seen, while the necrotic changes become visible only after 12 h. The cells die, thus, long before any morphological changes typical of necrosis can be detected. The term *cell death "by necrosis,"* which implies that necrotic changes accompany cell death, appears to be contradictory and confusing. Additionally confusing is the traditional use of the term *necrosis* in pathology to describe gross tissue changes, visible by eye and occurring after cell death, i.e., attributed to postmortem cell changes.

Majno and Joris (67) propose the term *accidental cell death* to define the mode of cell death which is now generally denoted as cell necrosis, and oncosis to portray the early stages of accidental cell death (Fig. 3). The term

FIG. 2. Morphological changes in cells undergoing apoptosis. **a,b:** Three-dimensional reconstruction of the nonapoptotic (a) and apoptotic cell nucleus (b) based on confocal microscopy of the PI-stained cells. HL-60 cells were induced to apoptosis by treatment with 1 $\mu\text{g}/\text{ml}$ of actinomycin D for 24 h. Following fixation in 70% ethanol and treatment with RNase the cells were stained with 1 $\mu\text{g}/\text{ml}$ of PI and their fluorescence was measured by flow cytometry. Cells with full DNA content (a) and fractional DNA content (b) were sorted, placed in glycerol on a microscope slide and imaged using a Zeiss LSM-10 fluorescence laser scanning microscope. Fifty sequential two-dimensional images were volume rendered to produce this three dimensional image using a Thinking Machine Corp. CM-200 supercomputer and the data processing resources of the Los Alamos National Laboratory Advanced Computing Laboratory (photographs kindly provided by T.M. Yoshida, B.L. Marrone and C. Hansen, Los Alamos National Laboratory). **c:** The "comet" assay (73) of apoptosis. To induce apoptosis exponentially growing HL-60 cells were incubated with 0.15 μM DNA topoisomerase I inhibitor camptothecin (CAM) for 4 h, fixed in 70% ethanol, then treated with RNase and suspended in 1% agarose film on the microscope slide and subjected to 5 min electrophoresis under conditions as described (37). The cells were counterstained with 1 $\mu\text{g}/\text{ml}$ of PI and viewed under fluorescence microscope (green light excitation). Note the typical "comet" appearance of DNA distribution from single cells, with the migrating low MW DNA fraction forming the comet's "tail". The integrated values of PI fluorescence in the "tail" regions represent a fraction of the degraded DNA while the total PI fluorescence ("tail" plus "head") represent total DNA content, correlating with the cell cycle position. **d,e:** Detection of apoptotic cells by staining with DAPI and sulforhodamine. HL-60 cells were induced to undergo apoptosis by treatment with 0.15 μM CAM for 4 h (41). The cytopspined cells were fixed for 15 min in 1% formaldehyde then in 70% ethanol, stained with 1 $\mu\text{g}/\text{ml}$ of DAPI and 20 $\mu\text{g}/\text{ml}$ sulforhodamine 101 in PBS and viewed either under UV light illumination (d) or under combined UV light and the transmitted light interference contrast illumination (e), as described (61). Note typical appearance of apoptotic cells having fragmented nuclei and the presence of individual apoptotic bodies (e, arrows). **f:** Increased sensitivity of DNA to denaturation in chromatin of apoptotic cells. HL-60 cells were induced to undergo apoptosis as in d

(above). The cytopspined cells were fixed in 70% ethanol, then sequentially treated with RNase A, 0.1M HCl, and stained with 6 $\mu\text{g}/\text{ml}$ AO at pH 2.6 (14,48). Note that DNA in chromatin of apoptotic cells stains more intensely in red compared to nonapoptotic cells which indicates its increased degree of single-strandedness (14). **g:** Increased sensitivity of DNA to denaturation in abnormal sperm cells. Smears of human sperm cells were fixed, treated with HCl, and stained with AO as in f (see refs. 27,43). The increase in DNA denaturability (red fluorescence) in abnormal spermatozoa (associated with infertility, refs. 27,28) correlates with extensive DNA breakage in these cells (43,84). Both changes are typical features of apoptosis, which suggests that apoptotic mechanisms play a role in elimination of a fraction of spermatozoa (possibly bearing genetic defects) at late stages of spermatogenesis from the reproductive pool, as discussed (43). **h:** Detection of DNA strand breaks in apoptotic cells. HL-60 cells were induced to undergo apoptosis by treatment with 0.15 μM CAM for 160 min. The cells were cytocentrifuged, fixed in 1% formaldehyde for 15 min, postfixed in 70% ethanol, and DNA strand breaks were labeled with BODIPY-conjugated dUTP (Molecular Probes, Inc., Eugene, OR) using exogenous TdT as described (42,61). Most apoptotic cells are at an early phase of apoptosis, prior to nuclear fragmentation. Note predominance of DNA strand breaks on periphery of the nucleus (arrows), which coincides with the areas where chromatin starts to condense during apoptosis. **i:** Simultaneous detection of apoptosis and DNA replication. HL-60 cells were pulse (1 h) labeled with BrdUrd then subjected to hyperthermia (43, 5°C; 30 min) to induce apoptosis and fixed 3 h thereafter. DNA strand breaks in apoptotic cells were labeled with CY-3-dCTP in the first sequence and the labeling was terminated by incubation with dideoxy-dUTP (ddUTP) to stop chain elongation. The cells were then illuminated with UV light and the photolytically generated DNA strand breaks (at sites of BrdUrd incorporation) were directly labeled with BODIPY-dUTP. The cells were then counterstained with sulforhodamine 101 and viewed under blue light illumination. With this combination of excitation and emission filters, the apoptotic cells show yellow fluorescence (CY-3) while BrdUrd incorporating cells fluoresce in green (BODIPY). A dim red fluorescence of cells unlabeled with CY-3 or BrdUrd (arrows) results from their counterstaining with sulforhodamine 101 (See ref. 61 for other details).

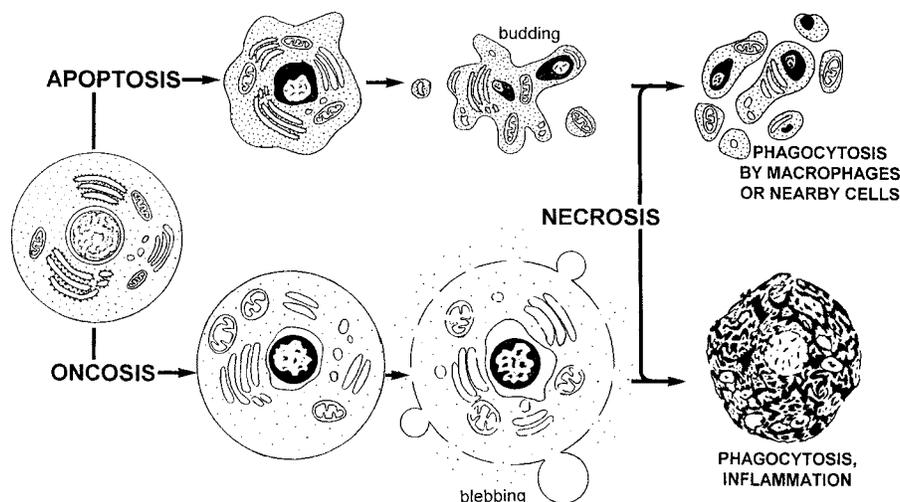


FIG. 3. Schematic representation of apoptosis, oncosis, and necrosis, according to taxonomy of cell death proposed by Majno and Joris (67). The early stages of apoptosis are characterized by a relatively intact plasma membrane and intracellular changes as described in the legend to Figure 1 and in the text. During the late stage (apoptotic necrosis) the plasma membrane transport function fails resulting in cells that cannot exclude trypan blue or PI, and the remains of the apoptotic cell are engulfed by

neighboring cells. During oncosis, cell mitochondria swell concomitant with a distortion of the mitochondrial structure and swelling of the whole cell. For some period of time, however, other vital cell functions are preserved albeit to different degrees. Rupture of the plasma membrane leads to a necrotic stage (oncotic necrosis) which is associated with local inflammation (modified, after Majno and Joris, ref. 67).

oncosis has been used by pathologists to describe cell death associated with cell swelling (*oncos* = "swelling"), which occurs during slow ischemia, e.g., as in the case of death of osteocytes entombed in the bone during bone formation (67). The features of oncosis are identical to those seen in the early phase of accidental cell death in a variety of cell systems. Within the framework of classification of cell death proposed by Majno and Joris (67), the necrotic step follows either oncosis or apoptosis and thus can be denoted either as oncotic or apoptotic "necrosis," respectively. The common features of such late necrotic cells, regardless of whether they were dying by apoptosis or oncosis, are loss of plasma membrane transport (ability to exclude charged dyes such as PI or trypan blue), autolytic processes, dissolution of the remnants of chromatin (karyolysis), etc. According to the terminology which we propose in this article, the term *cell necrobiology* may refer to the studies of the events which occur during both apoptosis and oncosis, while *cell necrology* refers to the postmortem events, defined by Majno and Joris as *necrosis*. The taxonomy of cell death proposed by these authors (67) is rational and has attractive elements which may clarify some inconsistencies in the current classification as discussed above.

It should be mentioned, however, that in contrast to apoptosis, which was studied extensively, little is known about characteristic features of early necrotic ("oncotic") cells, which may serve as markers for their identification by flow cytometry. Since plasma membrane integrity is largely preserved in both instances, the membrane permeant probes may not distinguish between early necrotic and apoptotic cells. One expects, however, that the mitochondrial swelling seen early during necrosis, is

associated with altered mitochondrial function. The latter can be detected by mitochondrial probes such as rhodamine 123 or cyanine dyes (17).

CAUSES OF INTEREST IN APOPTOSIS

As mentioned, cell necrobiology has recently become a focus of attention of researchers from a variety of disciplines. The critical development which stimulated this wide interest was realization that apoptosis, whether it occurs physiologically or is a manifestation of a pathological state, is an active and regulated mode of cell death. The regulation consists of several checkpoints at which a plethora of interacting molecules either promote or prevent apoptosis (74,101). A possibility thus exists for intervention, to interact with the regulatory machinery and thereby modulate the cell propensity to respond to the intrinsic or exogenous signals by death. Such a possibility is of obvious interest in oncology. Strategies for modulation of the sensitivity of tumor and/or normal cells to antitumor agents, via the regulatory mechanism of apoptosis, to increase efficiency of the treatment, and to lower toxicity to the patient are currently being explored in many laboratories worldwide (15,22,31,46). Recognition of the gene protecting cells from apoptosis (*bcl2*) as an oncogene (98) made it clear that not only the change in rate of cell proliferation but also the loss of their ability to die on schedule may be a cause of cancer. It also became apparent that tumor progression and the increase in malignancy may be associated with the change in propensity of tumor cells to undergo spontaneous apoptosis (45,50,56). Of interest in oncology, therefore, is the prognostic value of the rate of spontaneous apoptosis in tumors as well as of apoptosis induced by the treatment. In

the latter case, the effects of antitumor agents in terms of induction of apoptosis can be analyzed in the course of therapy, thereby providing a possibility of rapid assessment of their efficacy (38,60,89).

Immunology is another discipline where apoptosis is of great importance. This mode of cell death plays a fundamental role in clonal selection of T cells and is implicated as the key event in many other normal and pathological reactions (9,63). In particular, the mechanism of cell killing by natural killer (NK) lymphocytes is based on use of the apoptotic effectors machinery (106). Furthermore, since progression of AIDS appears to be correlated with the rate of T cells apoptosis, attempts are being made to monitor apoptosis of these cells as a sensitive marker of disease progression (23,68).

The key role of programmed cell death in tissue and organ development was recognized very early by embryologists. Apoptosis plays a role not only in normal tissue and organ modelling during embryogenesis, but likely is also triggered by environmental toxins, in which case it may be ectopic or unscheduled, leading to congenital malformations.

Male fertility is still another field where apoptosis appears to be of particular interest. It has been previously observed that DNA in chromatin of abnormal infertile sperm cells is, in contrast to normal sperm cells, very sensitive to heat or acid induced denaturation (ref. 27; Fig. 2g). This feature resembles very much DNA in chromatin of apoptotic cells (48,49; Fig. 2f). A correlation has been later observed between the increased DNA denaturability in infertile sperm cells and the presence of extensive DNA breakage, which is another feature of apoptosis (43). It was proposed, therefore, that apoptosis may be triggered to eliminate cells bearing, e.g., DNA mutations, even at late stages of spermatogenesis (43). However, in cells differentiated to such an extent, many apoptotic effectors may already be absent. Therefore, apoptosis in these cells may be incomplete, resulting only in activation of endonuclease which causes massive DNA degradation and elimination of the cell in terms of its reproductive capacity. The reproductively inactive spermatozoa may still have mitochondrial activity, normal motility and, in some cases, even normal morphology (27). It should be stressed that the increased *in situ* DNA denaturability in sperm cells, assayed by flow cytometry, has become extensively used as a marker of infertility and in toxicology studies to assay the genotoxic effects of environmental agents (28,84).

Flow cytometry has become a method of choice for analysis of apoptosis in a variety of cell systems (16,95). Methods chapters on the use of flow cytometry to analyze the modes of cell death have been recently published (18–20). A variety of flow cytometric methods have been developed, and new methods and modifications of established assays are being introduced at a rapid pace. The present article provides background information on apoptosis and on applicability of the cytometric methods in this

field, as well as updates the earlier reviews on this subject (16,95).

LIGHT SCATTERING PROPERTIES OF DYING CELLS

A cell traversing through the focus of the laser beam in a flow cytometer scatters the laser light. Analysis of the scattered light provides information about the cell size and structure (85). The intensity of light scattered in a forward direction correlates with cell size. The intensity of scattered light measured at a right angle to the laser beam (side scatter), on the other hand, correlates with granularity, refractiveness, and the presence of intracellular structures that can reflect the light (85). The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane, and in the case of apoptosis, chromatin condensation, nuclear fragmentation, and shedding of apoptotic bodies.

Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents. During apoptosis, on the other hand, the decrease in forward light scatter (which is a result of cell shrinkage) is not initially paralleled by a decrease in side scatter. Actually, a transient increase in right angle scatter can be seen during apoptosis in some cell systems (93). This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. In later stages of apoptosis, however, the intensity of light scattered at both, forward and right angle directions, is decreased (75,93).

The assay of cell viability by light scatter measurement is simple and can be combined with the analysis of surface immunofluorescence, e.g., to identify the phenotype of the dying cell. It can also be combined with functional assays such as mitochondrial potential, lysosomal proton pump, exclusion of PI or plasma membrane permeability to such dyes as Hoechst 33342 (HO342), as will be described further in this article.

The light scatter changes, however, are not specific to apoptosis. Mechanically broken cells, isolated cell nuclei, and necrotic cells also have diminished ability to scatter light. Identification of apoptosis or necrosis by light scatter, therefore, requires additional controls, and should be accompanied by another, more specific assay.

CHANGES IN THE PLASMA MEMBRANE

Features distinguishing dead from live cells include loss of transport function across the plasma membrane and often even the loss of its structural integrity. A plethora of assays of cell viability have been developed based on changes in the properties of the plasma membrane. Because the intact membrane of live cells excludes charged cationic dyes such as trypan blue, propidium or ethidium, and 7 amino-actinomycin-D (7-AMD), short incubation with these dyes results in selective labeling of dead cells, while live cells show minimal dye uptake (3,51,66,79,86,87,108).

The assays based on exclusion of these fluorochromes are commonly used to probe cell viability. Generally, a short (5–10 min) cell incubation in the presence of these fluorochromes labels dead cells, i.e., the cells that cannot exclude the dye (necrotic and late apoptotic cells, i.e., all “necrotic” cells according to the terminology proposed by Majno and Joris [ref. 67; Fig. 3]). The propidium exclusion test is frequently used as the flow cytometric equivalent of the trypan blue exclusion assay. These assays can be combined with analysis of cell surface immunophenotype (e.g., 66,87).

During apoptosis the membrane transport function becomes transiently defective prior to total loss of the ability to exclude these charged fluorochromes. At that stage of apoptosis, therefore, the rate of uptake of several of these fluorochromes has been shown to be increased, compared to control cells, and it is possible to differentiate populations of necrotic cells showing intensive fluorochrome labeling after a short incubation with the dye, from the moderately labeled apoptotic cells and from the nonapoptotic, live cells exhibiting minimal fluorochrome uptake (3,66,79).

The fluorochrome ethidium monoazide (EMA), like ethidium or propidium iodide, also is a positively charged molecule and is excluded from live and early apoptotic cells. It stains cells which have lost the integrity of their plasma membrane, i.e., necrotic and late apoptotic, as well as mechanically damaged cells (82). This dye can be photochemically cross-linked to nucleic acids by exposure to visible light. Cell incubation with EMA, followed by their illumination, irreversibly labels the cells which were unable to exclude the dye during incubation. The photolabeling of EMA can be conveniently combined with membrane immunophenotyping (92).

Another assay of membrane integrity employs the non-fluorescent esterase substrate, fluorescein diacetate (FDA). This substrate, after being taken up by live cells is hydrolyzed by intracellular esterases which are ubiquitous to all types of cells (83). The product of the hydrolysis, fluorescein, is a highly fluorescent, charged molecule which becomes trapped in intact cells. Incubation of cells in the presence of both propidium iodide (PI) and FDA, thus, labels live cells green (fluorescein) and dead cells red (PI). This is a convenient assay, widely used in flow cytometry (19).

Another DNA fluorochrome, Hoechst 33342 (HO342), unlike PI, is not excluded by live or apoptotic cells. Actually, it has been observed that short exposure of cells to low concentrations of HO342 led to strong labeling of apoptotic cells (3,8,21,76,78). Live cells, on the other hand, required much longer incubation with HO342 to obtain a comparable intensity of fluorescence. Supravital uptake of HO342 combined with exclusion of PI (to identify necrotic and late apoptotic cells) and with analysis of the cell's light scatter properties has been proposed as an assay of apoptosis (3,78). Hoechst fluorochrome HO258 appears to offer an advantage of increased stability of fluorescence, compared to HO342 (23). Other dyes, such as SYTO-16 and LCS-751, can also be used to discriminate

apoptotic cells, using the same principle as HO342, and so offer a choice of fluorochromes with different excitation and emission spectra (33). A combination of cell labeling with HO342 and 7-AMD was shown to discriminate between apoptotic and necrotic cells and to allow one to reveal the surface immunophenotype (86,87).

The degree of change in permeability of the plasma membrane to either charged or uncharged fluorochromes varies with the stage (advancement) of apoptosis, cell type, and mode of induction of apoptosis (e.g., DNA damage vs. engagement of the Fas receptor). Therefore, the optimal conditions for discrimination of apoptotic cells (fluorochrome concentration, time and temperature of incubation, and often ionic composition of the incubation medium) may significantly vary between different cell systems. Pilot experiments, therefore, are always necessary to customize the conditions for different cell systems for maximal discrimination of apoptotic from live, and/or from necrotic cells.

In live cells plasma membrane phospholipids are asymmetrically distributed between inner and outer surfaces of the plasma membrane. Thus, while phosphatidylcholine and sphingomyelin are exposed on the external surface of the lipid bilayer, phosphatidylserine is located on the inner surface (29). It has been shown recently that loss of phospholipid asymmetry leading to exposure of phosphatidylserine on the outside of the plasma membrane, is an early event of apoptosis (29,55). The anticoagulant annexin V preferentially binds to negatively charged phospholipids such as phosphatidylserine. By conjugating fluorescein to annexin V, it has been possible to use such a marker to identify apoptotic cells by flow cytometry (4a,55). During apoptosis the cells become reactive with annexin V after the onset of chromatin condensation but prior to the loss of the plasma membrane's ability to exclude PI. Therefore, by staining cells with a combination of fluoresceinated annexin V and PI, it is possible to detect nonapoptotic live cells (annexin V negative/PI negative), early apoptotic cells (annexin V positive, PI negative) and late apoptotic or necrotic cells (PI positive) by flow cytometry (55).

A rapid loss of plasma membrane structures such as pseudopodia and microvilli, resulting in the smooth appearance of the cell surface under the electron or phase contrast microscope, characterizes changes that occur relatively early during apoptosis (53,67,104,105). F-actin is a major constituent of pseudopodia. The phallotoxins are toxic cyclic peptides which bind to F-actin and prevent its depolymerization. Fluoresceinated phallotoxins are used as a probe of F-actin. It has been recently reported that the ability of cells to bind fluoresceinated phalloidin is lost during apoptosis (26). It was proposed, therefore, to combine cell staining with fluoresceinated phalloidin with DNA content analysis, to identify apoptotic cells and to reveal the cell cycle position of both, apoptotic and nonapoptotic cell populations (26). This approach was tested on HL-60 cells induced to undergo apoptosis by etoposide.

ANALYSIS OF CELL ORGANELLES

Several cell viability assays are based on functional tests of cell organelles. For example, the charged cationic green fluorochrome rhodamine 123 (Rh123) accumulates in mitochondria of live cells as a result of the transmembrane potential (52). Cell incubation with Rh123 results in labeling of live cells while dead cells, having uncharged mitochondria, show minimal Rh123 retention. Cell incubation with both Rh123 and PI labels live cells green (Rh123) and dead cells red (17). A transient phase of cell death, most likely by necrosis, however, can be detected, when the cells partially lose the ability to exclude PI and yet stain even more intensively than intact cells with Rh123. This suggests that mitochondrial transmembrane potential is transiently elevated early during necrosis, at the time of cell swelling and prior to rupture of the plasma membrane (17).

An early event of apoptosis is a decrease in mitochondrial transmembrane potential which is reflected by a loss of the cell's ability to accumulate Rh123 (89a) or cyanine dyes in mitochondria (4a,44a,55,78a). This event is associated with an increased production of superoxide anions (reactive oxygen intermediates) and increased content of the reduced form of cellular glutathione (4a, 44a). The product of the *bcl2* gene appears to play a critical role preventing the loss of the mitochondrial transmembrane potential during apoptosis (4a).

Other organelles which can be probed by flow cytometry are lysosomes. Incubation of cells in the presence of 1–2 µg/ml of the metachromatic fluorochrome acridine orange (AO) results in the uptake of this dye by lysosomes of live cells which fluoresce red (97). The uptake is the result of an active proton pump in lysosomes: the high proton concentration (low pH) causes AO, which can enter the lysosome in an uncharged form, to become protonated and thus entrapped in the organelle. Dead cells, at that low AO concentration, exhibit weak green and minimal red fluorescence. This assay is useful for cells that have numerous active lysosomes, such as monocytes, macrophages, etc.

It should be stressed here again that because, in the early stages of apoptosis, both the plasma membrane is preserved and most organelles and cellular functions remain relatively unchanged compared to live cells, the assays based on the function of cell organelles, like the dye exclusion tests, are not always able to identify cells that die by apoptosis. However, they are well suited to discriminate between early apoptotic and live cells versus necrotic cells (or between apoptotic and oncotic cells versus "necrotic" cells, according to the terminology of Majno and Joris, ref. 67; Fig. 3).

SENSITIVITY TO DENATURATION OF DNA IN SITU

The sensitivity of DNA in situ to denaturation can be measured based on the metachromatic property of the fluorochrome AO. Under proper conditions this dye can differentially stain double-stranded (ds) vs. single-stranded (ss) nucleic acids (14). Namely, when AO intercalates into ds DNA it emits green fluorescence. In contrast, the

products of AO interaction with ss DNA fluoresce red. In this method, the cells are briefly pre-fixed in formaldehyde followed by ethanol postfixation. Cell fixation abolishes staining of lysosomes, as described above in this article. Following removal of RNA from the fixed cells by their preincubation with RNase, DNA is partially denatured in situ by short cell exposure to acid. The cells are then stained with AO at low pH to prevent DNA renaturation. It was shown before that the sensitivity of DNA in situ to denaturation is higher in condensed chromatin of mitotic cells compared to the noncondensed chromatin of interphase cells (14). Apoptotic cells, like mitotic cells, have a larger fraction of DNA in the denatured form, and more intense red and reduced green fluorescence, compared to nonapoptotic (interphase) cells; the latter stain strongly green but have low red fluorescence (48,49; Fig. 2f,g). An increased degree of DNA denaturation (single-strandedness) in apoptotic cells also can be detected immunocytochemically, using an antibody reactive with ssDNA (32).

The methods based on sensitivity of DNA to undergo denaturation (14,48) may be uniquely applicable in situations where internucleosomal DNA degradation is not apparent (1,5,10,11,107), and thus when other methods of apoptotic cells identification (e.g., the ones that rely on detection of DNA degradation) may fail. They cannot, however, discriminate between mitotic and apoptotic cells. However, the method utilizing AO has been extensively applied to identify abnormal (apoptotic-like) sperm cells from individuals with reduced fertility and to study genotoxic effects of antitumor drugs and environmental poisons during spermatogenesis (27,28,84).

MEASUREMENT OF CELLULAR DNA CONTENT

Extensive DNA cleavage, being a characteristic feature of apoptosis, provided a basis for the development of novel flow cytometric assays to identify apoptotic cells. Two approaches are frequently used to detect DNA cleavage. One is based on extraction of low MW DNA prior to cell staining. The other relies on fluorochrome labeling of DNA strand breaks in situ.

In the first approach, the cellular DNA content is measured following cell permeabilization with detergents or prefixation with precipitating fixatives such as alcohols, or acetone. Cell permeabilization or alcohol fixation does not fully preserve the degraded DNA within apoptotic cells: this fraction of DNA leaks out during subsequent cell rinsing and staining. As a consequence, apoptotic cells contain reduced DNA content and therefore can be recognized, following staining of cellular DNA, as cells with low DNA stainability ("sub-G₁" peak), less than that of G₁ cells (24,25,71,100).

The degree of DNA degradation varies depending on the stage of apoptosis, cell type and often the nature of the apoptosis-inducing agent. The extractability of DNA during the staining procedure (and thus separation of apoptotic from live cells by this assay), also varies. It has been noted that addition of high molarity phosphate-citrate buffer to the rinsing solution enhances extraction of the degraded DNA (37). This approach can be used to control

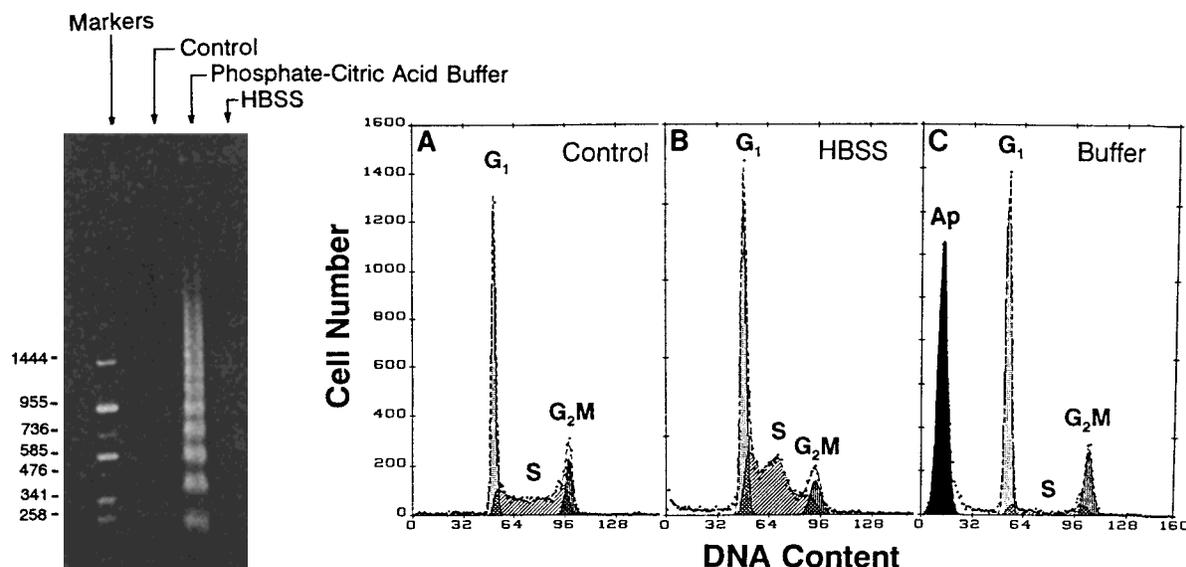


FIG. 4. Combined analysis of apoptosis by DNA gel electrophoresis and flow cytometry of the same cells. DNA gel electrophoresis and DNA content frequency histograms of corresponding HL-60 cells untreated (Control, A) and treated with 0.15 μ M DNA topoisomerase I inhibitor camptothecin which selectively induces apoptosis of S phase cells (B, C). The cells represented by B histogram were extracted with Hanks' balanced salt solution (HBSS) while those represented by C histogram by 0.2 M phosphate-citrate buffer at pH 7.8. DNA extracted with HBSS or

with the buffer was analyzed by gel electrophoresis, as shown in respective lanes, while the same cells after the extraction were stained with DAPI and subjected to flow cytometry. Note the presence of degraded DNA extracted from the camptothecin treated cells by the buffer and its absence in the HBSS extract and in the extract from untreated (control) cells. The DNA marker size is expressed in base pairs (reproduced with permission of the publisher from 37).

the extent of DNA extraction from apoptotic cells to the desired level to obtain the optimal separation of apoptotic cells by flow cytometry. Figure 4 illustrates an application of this approach to extract DNA from apoptotic cells and for electrophoretic analysis of this DNA extracted from the very same cells that were subsequently analyzed by flow cytometry (37).

Since measurement of DNA content provides information about the cell cycle position of the nonapoptotic cells, this approach can be applied to investigate the cell cycle specificity of apoptosis. Other advantages of this approach are its simplicity and applicability to any DNA fluorochrome or instrument. The combination of correlated DNA and RNA measurements, which allows one to identify G_0 cells, makes it possible to distinguish whether apoptosis is preferential to G_1 or G_0 cells (4).

The limitation of the DNA extraction approach is low specificity in the detection of apoptosis. The "sub G_1 " peak can represent, in addition to apoptotic cells, mechanically damaged cells, cells with lower DNA content (e.g., in a sample containing cell populations with different DNA indices) or cells with different chromatin structure (e.g., cells undergoing erythroid differentiation) in which the accessibility of DNA to the fluorochrome is diminished (25). As will be discussed further, this is of special concern when unfixed cells are lysed in hypotonic solution, resulting in isolation of multiple nuclear fragments. Hence, the number of "sub G_1 " cells in such a preparation represents the number of nuclear fragments and provides no information on the number of apoptotic cells. Furthermore, in

addition to nuclear fragments, individual chromosomes released from mitotic cells, cell debris, and micronuclei all may be mistakenly identified as apoptotic cells, especially when using logarithmic scale of DNA content for data accumulation and analysis (Fig. 5).

IN SITU LABELING OF DNA STRAND BREAKS

Endonucleolytic DNA cleavage results in the presence of extensive DNA breakage. The 3' OH termini in DNA breaks are detected by attaching to them biotin or digoxigenin conjugated nucleotides, in a reaction catalyzed by exogenous TdT ("end-labeling," "tailing," "TUNEL") or DNA polymerase (nick translation) (35,36,39-43,91, 103; Figs. 6-8). Fluorochrome conjugated avidin or digoxigenin antibody has often been used in the second step of the reaction to label DNA strand breaks. A simplified, single-step procedure has recently been developed, using fluorochromes directly conjugated to deoxynucleotides (62). A new method was recently introduced, in which BrdUTP, incorporated by TdT, is used as the marker of DNA strand breaks (59,61; Fig. 6). A similar approach has been used previously to probe the sensitivity of nuclear DNA to DNase I as a probe of nuclear structure (94). The method based on BrdUTP incorporation (59) is simpler, more sensitive, and costs less compared with the digoxigenin or biotin labeling. Commercial kits designed to label DNA strand breaks for identification of apoptotic cells are offered by ONCOR, Inc. (Gaithersburg, MD; double-step assay using digoxigenin) and Phoenix Flow Systems (San Diego, CA, single-step and BrdUTP labeling assays).

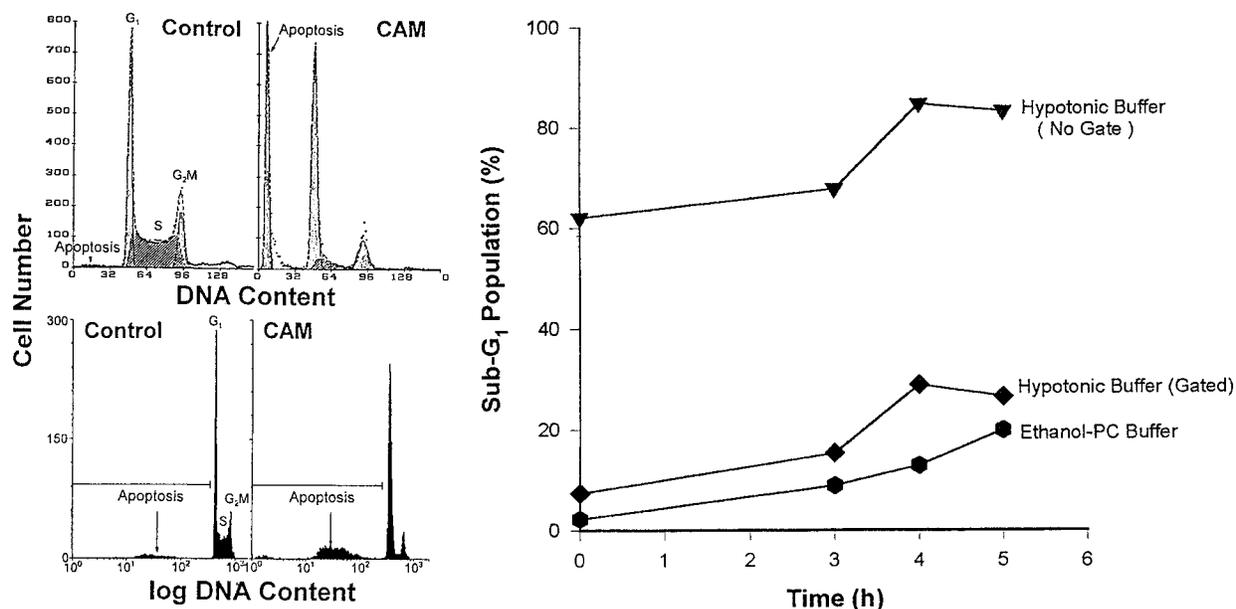


FIG. 5. DNA content frequency histograms and percent of cells with fractional DNA content in preparations of detergent treated vs. ethanol fixed cells. HL-60 cells were incubated with 0.15 μM camptothecin to for 3, 4, or 5 h to induce apoptosis of S phase cells, as described (39,42). The cells were then fixed in 70% ethanol, rinsed with 0.2 M phosphate-citrate (PC) buffer at pH 7.8, and stained with 1 $\mu\text{g}/\text{ml}$ DAPI in PBS (top histograms). Alternatively, following incubation with the drug the cells were suspended in hypotonic citrate buffer (0.1% Na-citrate) containing 1 $\mu\text{g}/\text{ml}$ DAPI and 0.1% Triton X-100 (bottom histograms). Linear scale was used for analysis of DNA content of fixed cells while the analysis of the hypotonic buffer treated (lysed) cells was performed using logarithmic scale (log amplifiers, bottom histograms). The percent of apoptotic cells (cells with fractional DNA content; sub-G₁ cells) was established after

The approach based on DNA strand break labeling in the assay employing TdT appears to be the most specific in terms of positive identification of apoptotic cells. Namely, necrotic cells or cells with primary breaks induced by ionizing radiation (up to the dose of 25 Gy of γ radiation), or DNA damaging drugs, have an order of magnitude fewer DNA strand breaks than apoptotic cells (39). Because cellular DNA content of not only nonapoptotic but also apoptotic cells is measured, the method offers a unique possibility to analyze the cell cycle position, and/or DNA ploidy, of apoptotic cells (ref. 41; Fig.7).

It has been reported that comparative labeling of DNA strand breaks utilizing DNA polymerase vs. TdT allows one to discriminate between apoptotic and necrotic cells (35). The difference in intensity of labeling of apoptotic and necrotic cells in these assays, however, was inadequate to fully separate these populations (35).

The procedure of DNA strand break labeling is rather complex and involves many reagents. Negative results, therefore, may not necessarily mean the absence of DNA strand breaks but may be a result of some methodological problems, such as the loss of TdT activity, degradation of triphosphonucleotides, etc. It is always necessary, therefore, to include a positive and negative control. An excellent control consist HL-60 cells treated (during their

deconvolution of the respective histograms. The estimate of apoptotic cells was obtained either within the light scatter gate (forward vs. side scatter) to exclude objects with minimal light scatter, or without the gating. The plot shows that the percent of the objects with fractional DNA content was many fold higher when unfixed, lysed cells were analyzed without the light scatter gate compared to the ethanol fixed cells. The percentage of such objects also was significantly higher when the objects with low light scatter values were excluded from the analysis ("Gated"). Analysis of fixed cells using logarithmic scale was not significantly different compared to that analysed linearly (not shown). The data indicate that cell lysis results in an overestimate of apoptotic index, most likely due to the erroneous identification of the nuclear fragments from apoptotic cells with minimal DNA content as single apoptotic cells.

exponential growth) for 3–4 h with 0.2 μM of the DNA topoisomerase I inhibitor camptothecin (CAM). Because CAM induces apoptosis selectively during S phase, the populations of G₁ and G₂/M cells may serve as negative populations (background), while the S phase cells in the same sample, serve as the positive control (Figs. 6, 7).

As shown in Figure 8, the detection of apoptotic cells based on the presence of DNA strand breaks can be combined with analysis of DNA replication (61). The advantage of this approach is that it offers a possibility, in a single measurement, to identify the cells which incorporated halogenated DNA precursors and the cells undergoing apoptosis, in relation to their DNA content.

CHOICE OF THE METHOD OF CELL DEATH ANALYSIS

The optimal method for identification of dead cells depends on the cell system, the nature of the inducer of cell death, the mode of cell death, the particular information that is being sought (e.g., specificity of apoptosis with respect to cell cycle phase or DNA ploidy) and the technical restrictions (e.g., the need for sample storage or transportation, type of flow cytometer available, etc.).

The methods based on analysis of plasma membrane integrity (exclusion of charged fluorochromes or FDA

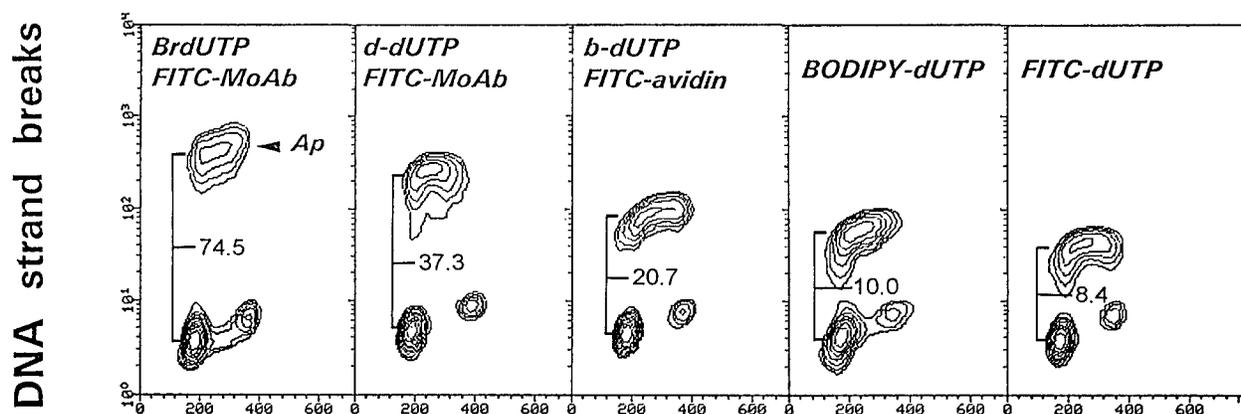


FIG. 6. Detection of DNA strand breaks in apoptotic cells by the methods utilizing different deoxynucleotide conjugates and direct as well as indirect labeling, in the reaction catalyzed by exogenous terminal transferase. Detection of apoptotic cells using different methods of DNA strand break labeling. HL-60 cells were treated with 0.15 μ M camptothecin for 3 h. The first three panels to the left represent indirect labeling of DNA strand breaks, utilizing either BrdUTP, digoxigenin conjugated dUTP

(d-dUTP) or biotinylated dUTP (b-dUTP). The two right panels show cell distributions following a direct, single-step DNA strand break labeling, either with BODIPY or FITC conjugated dUTP. The figures represent the mean fluorescence intensity ratio of the apoptotic S phase cells to non-apoptotic, G₁ cells and indicate that the greatest separation of apoptotic cells is achieved following DNA strand break labeling with BrdUTP. (With permission from the publisher, ref. 59).

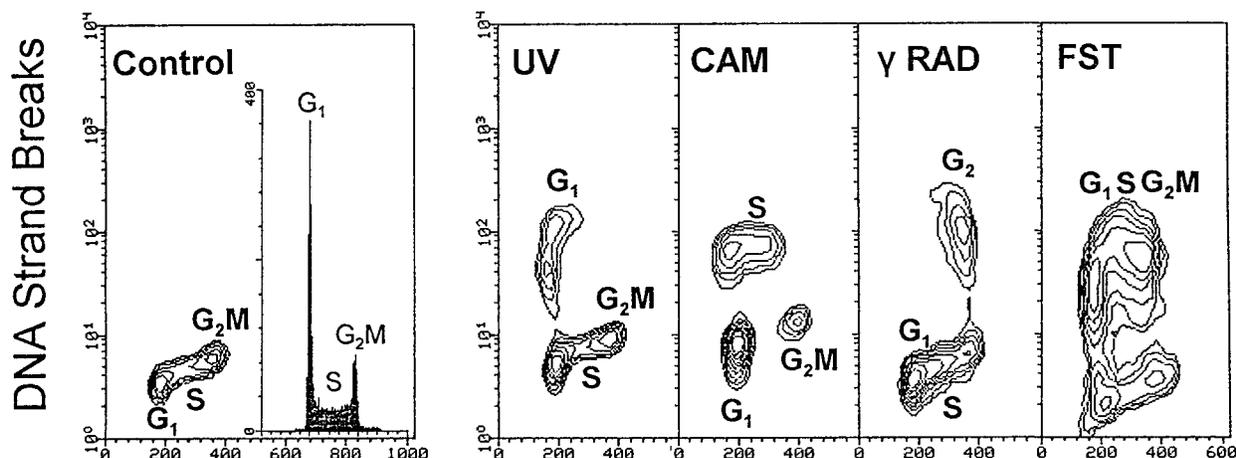


FIG. 7. Analysis of the cell cycle specificity of apoptosis by the method based on the in situ DNA strand break labeling. DNA strand break labeling was performed on HL-60 cells, untreated (control) as well as on the cells treated with UV light, 0.15 μ M camptothecin (CAM), γ radiation (3 Gy), or DNA topoisomerase II inhibitor fostriecin (FST), 4 h after the cells' exposure to drugs or irradiation, as described (41). DNA strand breaks

were labeled with digoxigenin-conjugated dUTP followed by FITC-digoxigenin MoAb. The cells were counterstained with PI. Note that UV light, CAM, or γ radiation selectively induce apoptosis of G₁, S, or G₂/M cells, respectively, while FST is less selective in terms of the cell cycle phase specificity. For details see (41).

hydrolysis), although simple and inexpensive, may fail to identify apoptotic cells, especially at early stages of apoptosis. They can be used to identify necrotic cells, cells damaged mechanically or cells advanced in apoptosis. Their major use is in enumeration of live cells in cultures (e.g., to exclude dead cells during analysis of cell growth curves, as when using the trypan blue exclusion assay), or in discrimination of mechanically broken cells (e.g., following mechanical cell isolation from solid tumors). Careful analysis of the kinetics of cell stainability with these dyes, however, may in some cell systems distinguish necrotic from apoptotic cells (66,87).

Positive identification of apoptotic cells is more difficult. The most specific assays appear to be based on the

detection of DNA strand breaks. The number of DNA strand breaks in apoptotic cells appears to be of such large magnitude that the intensity of DNA strand break labeling can be a specific marker of these cells (39). The situation, however, is complicated when dealing with atypical apoptosis, which, as discussed earlier, is characterized by the lack of internucleosomal DNA degradation. The number of DNA strand breaks in such atypical apoptotic cells is not always adequate for their identification by this method. The data of Chapman et al., (7), however, indicate that apoptotic cells can be distinguished by the DNA strand breaks assay even in the absence of internucleosomal DNA cleavage. On the other hand false positive recognition of apoptosis, by this method, may occur in situations where

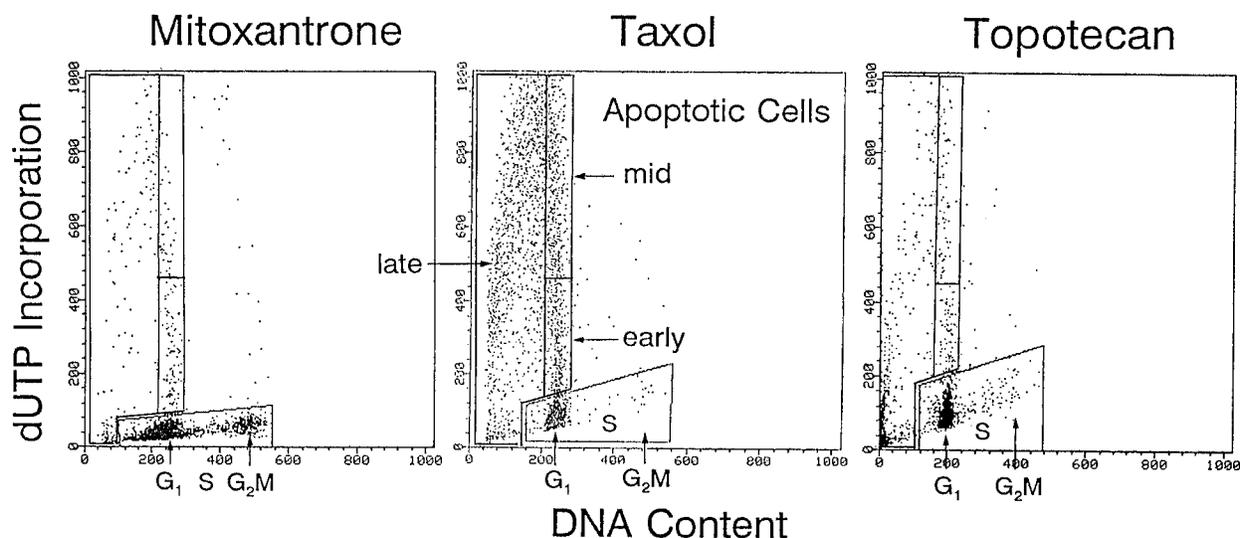


FIG. 8. Examples of apoptosis of leukemic cells from peripheral blood of the patients undergoing chemotherapy. The data show the presence of cells with DNA strand breaks in relation to their DNA content. The patients with acute leukemias were treated with Mitoxantrone, taxol, or DNA topoisomerase I inhibitor Topotecan and their blood cells were examined for DNA strand breaks as described (40,60,89). Note high

heterogeneity of cells with respect to the number of DNA strand breaks per cell, which reflects duration of apoptosis. After reaching maximum number of DNA strand breaks, cells start to lose DNA perhaps by shedding apoptotic bodies. With a loss of DNA, due to loss of the primers (3'OH ends of the DNA strand breaks) less labeled dUTP is incorporated per cell.

internucleosomal DNA cleavage accompanies necrosis (11).

Apoptosis can be identified with better assurance when more than a single viability assay is used. Thus, simultaneous assessment of plasma membrane integrity (e.g., exclusion of charged fluorochromes or hydrolysis of FDA), together with either membrane permeability (HO342 uptake), mitochondrial transmembrane potential (Rh123 uptake), DNA sensitivity to denaturation or DNA cleavage assays, offer a more certain means of identification of the mode of cell death than each of these methods alone.

Cost and simplicity play a role in the choice of the method. The least expensive and most rapid discrimination of apoptotic cells is based on DNA content analysis. This approach is routinely used in our laboratory for screening drug effects *in vitro*, in particular when large number of samples have to be measured. In addition to the enumeration of apoptotic cells offered by this method, the cell cycle specific effects can easily be recognized from DNA content histograms of the nonapoptotic cell populations. The data are then confirmed using the DNA strand break labeling assay.

Flow cytometry can provide rapid, quantitative, and objective assays of cell viability which may be applied for enumeration of apoptotic or necrotic cells. However, regardless of the particular method that has been used to identify the mode of cell death, flow cytometric analysis should always be confirmed by the inspection of cells under the light or electron microscope. Morphological changes during apoptosis are unique and they should be the deciding factor when ambiguity arises regarding the mechanisms of cell death. Furthermore, apoptosis was

originally defined based on the analysis of cell morphology (54). Morphological criteria of identification of apoptotic and necrotic cells, therefore, should be taken into an account in conjunction with flow cytometric analysis of cell death.

COMMON PITFALLS IN ANALYSIS OF APOPTOSIS BY FLOW CYTOMETRY

Use of Unfixed Cells and Detergent or Hypotonic Solutions in DNA Content Analysis

As described above, one of the most common methods of identification of apoptotic cells relies on DNA content analysis: Objects with a fractional DNA content are assumed to be apoptotic cells. This, however, holds true for cells which were prefixed with precipitating fixatives such as alcohols or acetone. It is quite common, however, that this analysis is performed on cells which were subjected to treatment with a detergent or hypotonic solution. Such treatment lyses the cell. Because the nucleus becomes fragmented during apoptosis and numerous individual chromatin fragments may be present in a single cell, the percentage of objects with a fractional DNA content (represented by the "sub-G₁" peak) released from a lysed cells does not correspond to the apoptotic index (Fig. 5). Furthermore, lysis of mitotic cells, or cells with micronuclei releases individual chromosomes or micronuclei, respectively. Individual chromosomes, chromosome aggregates, as well as micronuclei are all objects with a fractional DNA content which erroneously can be identified as apoptotic cells.

An exception to the rule of not using detergents is gentle permeabilization of the cell with the detergent but

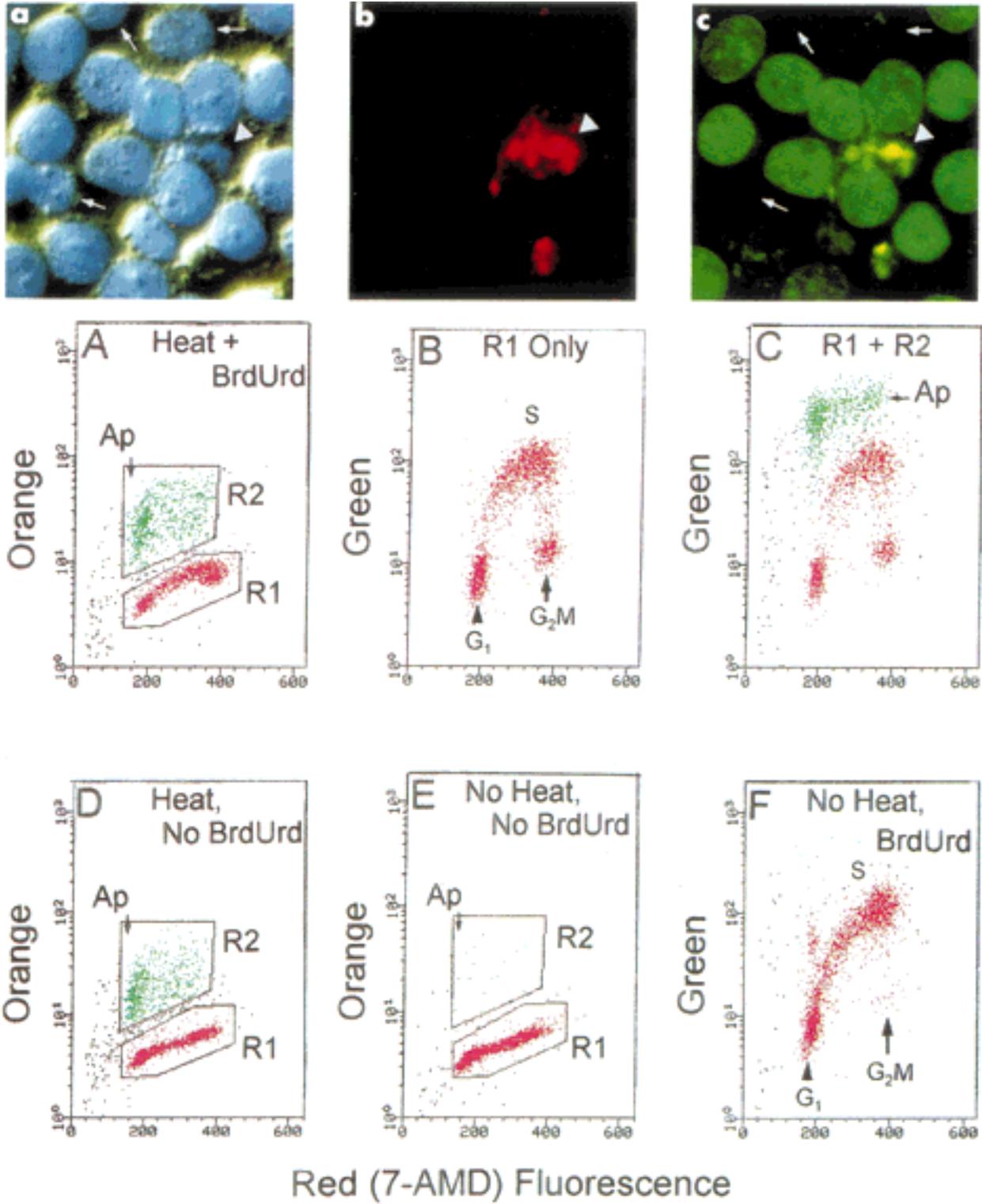


FIG. 9 (Legend on facing page).

in the presence of exogenous proteins such as serum or serum albumin. The presence of 1% (w/w) albumin or 10% (v/w) serum protects cells from lysis (e.g., induced by 0.1% Triton X-100) without affecting their permeabilization by detergent. In fact, this method is used for simultaneous analysis of DNA and RNA (4). Apoptotic or nonapoptotic cells suspended in saline containing the detergent and serum proteins, however, are extremely fragile and pipetting, vortexing or even shaking the tube containing the suspension, causes their lysis and release of the cell constituents into solution.

Use of Logarithmic Scale in DNA Content Analysis

It is also common to see a logarithmic scale (log amplifiers) used to measure and display DNA content when trying to quantitate apoptosis. Such use frequently parallels the use of detergents. A logarithmic scale allows one to measure and record objects with 1% or even 0.1% of the DNA content of intact, nonapoptotic cells. Most such objects cannot be apoptotic cells. In the case of cell lysis by detergents, as discussed above, these objects represent nuclear fragments, individual apoptotic bodies, chromosomes, chromosome aggregates or micronuclei (Fig. 5).

For standardization, it is advisable to identify apoptotic cells as the objects with a fractional DNA content which is no less than 10% (or 20%) of that of intact G_1 cells. This may result in an underestimate of apoptosis, but the bias is constant and is less of a danger compared to counting all objects with a fractional DNA content, for example ranging from 0.1% to 100% of intact G_1 cells. In the latter case cellular debris, single chromosomes from broken mitotic cells, chromosome clumps, contaminating bacteria, etc., all having very low DNA content, and may be erroneously classified as apoptotic cells. Application of a linear rather than a logarithmic scale provides better assurance that objects with a minimal DNA content are excluded from the analysis.

Assumption That Percentage of Apoptotic Cells Represents the Rate of Cells Dying by Apoptosis

Similar to mitosis, apoptosis is of short duration. It is also of variable duration. The time window during which apoptotic cells demonstrate their characteristic features that allow them to be identifiable varies depending on the method used, cell type, or nature of the inducer of apoptosis. Some inducers may slow down or accelerate the apoptotic process by affecting the rate of formation and shedding of apoptotic bodies, endonucleolysis, proteolysis, etc., thus altering the duration of the "time window" by which we identify the apoptotic cell. One has to keep in mind, for example, that an observed twofold increase in apoptotic index may either indicate that twice as many cells were dying by apoptosis, compared to control, or that the same number of cells were dying but that the duration of apoptosis was prolonged twofold. Unfortunately, no method exists to obtain cumulative estimates of the rate of cell entrance to apoptosis as there is, for example, for mitosis, which can be arrested by microtubule poisons in a stathmokinetic experiment. In short, the percentage of apoptotic cells in a cell population estimated by a given method is not a measure of the rate of the cells dying by apoptosis.

To estimate the rate (kinetics) of cell death the absolute number (not the percentage) of live cells should be measured in the culture, together with the rate of cell proliferation. The latter may be obtained from the rate of cell entrance to mitosis (cell "birth rate") in a stathmokinetic experiment. The observed deficit in the actual number of live cells from the expected number of live cells estimated based on the rate of cell birth provides the cumulative measure of cell loss (death). Indirectly, the cell proliferation rate can be inferred from the percentage of cells incorporating BrdUrd or from the mitotic index, under the assumption that the treatment which induces apoptosis does not affect the duration of any particular phase of the cell cycle (generally a risky assumption). The

FIG. 9. Simultaneous detection of apoptosis and DNA replication. a-c: Exponentially growing HL-60 cells were incubated for 1 h in the presence of BrdUrd to label DNA replicating cells. The cells were fixed and DNA strand breaks in apoptotic cells (approximately 1-5% cells undergoes spontaneous apoptosis in HL-60 cell cultures) were labeled with dCTP conjugated to CY3 followed by ddUTP to terminate chain elongation. The cells were then illuminated with UV light and the photolytically generated DNA strand breaks were subsequently labeled with BODIPY-dUTP. The cells were counterstained with 1 μ g/ml of DAPI. a: mixed illumination, incident UV light plus transmitted light (interference contrast). All cells are seen, DNA stains blue with DAPI. b: the same field, green light fluorescence excitation. Only red component of the CY-3 fluorescence is seen (arrowhead, apoptotic cell, spontaneous apoptosis). c: the same field, fluorescence excitation with blue light. The BODIPY labeled (BrdUrd incorporating cells) show green fluorescence. Also the yellow component of the CY-3 fluorescence of the apoptotic cell is apparent (arrowhead). Note the presence of cells which did not incorporate BrdUrd (arrows in a and c). See (61) for details. A-F: Flow cytometric simultaneous detection of DNA replicating and apoptotic cells. HL-60 cells were incubated with BrdUrd for 30 min. The cells were then rinsed, suspended in medium lacking BrdUrd, and, to induce apoptosis, subjected to hyperthermia (30 min at 43.5°C) After additional incubation at 37°C for 3 h the cells were fixed and then subjected to DNA strand break labeling. In the first step, the apoptosis-associated DNA breaks were labeled with

biotin-conjugated dUTP followed by ddUTP (to stop the chain elongation) and streptavidin PE conjugate. The cells were then exposed to UV light to photolyze DNA containing incorporated BrdUrd and the UV light induced DNA breaks were labeled, in the second step, with the antiBrdUrd MoAb conjugated to FITC. Cellular DNA was counterstained with 7AMD and the cells were analyzed by single laser flow cytometry (FACScan, Becton Dickinson). A: Orange-yellow fluorescence (detector #2) primarily of PE allows to discriminate apoptotic cells (R2, Ap, green dots). B: The population of apoptotic cells was gated out as shown in panel A and the cells in gate R1 were plotted according to their green fluorescence. Note the presence of S phase cells (DNA index between 1.0 and 2.0) showing green fluorescence (detector #1). C: Plot as in B, except that the population in R2 was not gated out. Note that apoptotic cells show high green fluorescence, which to a large extent is due to the strong green component of PE emission spectrum (No electronic compensation of the emission spectra was used). D: Control, the cells were treated as in A except labeling with BrdUrd. E: Control, the cells were treated as in A except they were neither heat treated nor BrdUrd labeled. F: The cells were treated as in C, except not subjected to hyperthermia. Compared with C, note paucity of apoptotic cells and advancement of the BrdUrd labeled cells through S and G_2/M during the 3-h postincubation period: most labeled cells are in late S and G_2/M , some even had divided (G_1). See (61) for further details of cell labeling and analysis.

latter approach, however, cannot provide an estimate of the cell birth rate.

Assumption That Apoptotic Cells Must Exhibit All Typical (Classical) Features of Apoptosis

The lack of evidence of apoptosis, detected by a particular method, is not evidence of the lack of apoptosis. As already discussed, there are numerous examples in the literature where cells die by a process resembling apoptosis which lacks one or more typical apoptotic features. Most frequently, DNA degradation stops after creation of 50–300-kb fragments, meaning there is no internucleosomal fragmentation seen and, therefore, fewer in situ DNA strand breaks compared with classical apoptosis (72). The method of identification of apoptosis based on detection of the missing feature (e.g., DNA laddering on gels) fails to identify atypical apoptosis in such a situation.

Application of more than one method, each based on a different principle (i.e., detecting a different cell feature) stands a better chance of detecting atypical apoptosis than any single method. One expects, for example, that if DNA in apoptotic cells was fragmented to 50–300 kb it would not be extractable and, as a result, such cells could not be identified as apoptotic either by the method based on analysis of DNA content or that based on “laddering” DNA during electrophoresis. However, the presence of in situ DNA strand breaks, even at a lower frequency than in the case of typical apoptosis, can be used as a marker in such cells (7). It is likely (though not yet proven) that such apoptotic cells may also be recognized based on their reduced F-actin stainability with FITC-phalloidin (26) or by the FITC-annexin V conjugate reacting with cell surface phosphatidylserine (55).

Incorrect Identification of Late Apoptotic Cells as Necrotic Cells

Cells very advanced in apoptosis resemble necrotic cells to such an extent that, as mentioned earlier in this article, the term *apoptotic necrosis* was proposed to define the late stages of apoptosis (ref. 67; see Fig. 3). Because the ability of apoptotic cells to exclude charged cationic fluorochromes such as PI or 7-AMD is lost at these later stages, the discrimination between late apoptosis and necrosis cannot be accomplished by methods utilizing these dyes. Since phosphatidylserine may be accessible to annexin V in necrotic cells due to the fact that the integrity of the plasma membrane is compromised, it is expected that this assay also cannot be used to identify apoptotic from necrotic cells. Other methods, therefore, should be used. Extensive DNA fragmentation detected by DNA gel electrophoresis and analysis of cellular DNA content, or in situ presence of numerous DNA strand breaks may serve as markers to distinguish late apoptotic from necrotic cells.

Selective Collection of Adherent Cells From Tissue Culture Flasks

Relatively early during apoptosis cells detach from the surface of the culture flasks and float in the medium. The standard procedure of discarding the medium, trypsiniza-

tion or EDTA-treatment of the attached cells and their collection, results in selective loss of apoptotic cells. Such loss may vary from flask to flask depending on handling the culture, e.g., degree of mixing or shaking, efficiency in discarding the old medium, etc. Surprisingly, cell trypsinization and discarding the medium is quite a common practice in studies of apoptosis, as is evident from many published papers (which are not cited here to avoid embarrassing the authors). Needless to say, such an approach can not be used for quantitative analysis of apoptosis. To estimate the apoptotic index in cultures of adherent cells, it is essential to add the floating cells to the trypsinized ones and measure them together.

Similarly, density gradient (e.g., using Ficoll-Hypaque solutions) separation of the cells may result in selective loss of dying and dead cells. The knowledge of any selective loss of dead cells in cell populations purified by such approaches is essential when one is studying apoptosis.

Use of the New Untested Commercial Kits

A number of commercial kits have recently become available to detect apoptosis and many reagent companies are racing to introduce their “unique apoptosis detection kits.” Some of these kits have solid experimental foundations and have been successfully tested on a variety of cell systems. Other kits, however, especially those advertised by vendors who do not fully explain the principle of detection of apoptosis on which the kit is based, and do not list its chemical composition, may not be universally applicable. Unfortunately, it is a common practice for some vendors to test only a single cell line using a single agent to trigger apoptosis (generally, either a leukemic cell line treated with the Fas ligand, or HL-60 cells treated with a DNA topoisomerase inhibitor). Yet the claim is often made that their kit is applicable to different cell systems. Before application of any new kit it is advisable to confirm that at least 3–4 independent laboratories have already successfully used it on different cell types. Furthermore, it is good practice to use the new kit in parallel with an well-established methodology, in a few experiments. This would allow one, by comparison of the apoptotic indices, to estimate the time-window of detection of apoptosis by the new method, compared to the one which is already established and accepted in the field.

Use of Flow Cytometry Without Morphological Examination of the Sample

Apoptosis was originally defined as a specific mode of cell death based on very characteristic changes in cell morphology (54). Although individual features of apoptosis may serve as markers for detection and analysis of the proportion of apoptotic cells in the cell populations studied by flow cytometry, the mode of cell death always should be positively identified by inspection of cells by light or electron microscopy. Therefore, when analysis is done by flow cytometry and any ambiguity arises regarding the mechanism of cell death, the morphological

changes should be the deciding attribute in resolving the uncertainty.

It should be stressed that optimal preparations for light microscopy require cytopspinning of live cells following by their fixation and staining on slides. The cells are then flat and their morphology is easy to assess (Fig. 2). On the other hand, when the cells are initially fixed and stained in suspension, then transferred to slides and analyzed under the microscope, their morphology is obscured by the unfavorable geometry: the cells are spherical and thick and require confocal microscopy to reveal details such as early signs of apoptotic chromatin condensation.

We have noticed that differential staining of cellular DNA and protein with DAPI and sulforhodamine 101 of the cells on slides, which is very rapid and simple, gives a very good morphological resolution of apoptosis and necrosis (Fig. 2d,e). Other DNA fluorochromes, such as PI or 7-AMD, or the DNA/RNA fluorochrome AO, can be used as well.

ROLE OF FLOW CYTOMETRY IN CELL NECROBIOLOGY

The explosive growth in recent years of flow cytometry for the analysis of cell death in a variety of disciplines of biology and medicine is the best evidence of the value of this methodology in cell necrobiology. Two general directions characterize this growth. One direction is the use of flow cytometry to quantify apoptotic cells (apoptotic index). Compared to the alternative methods (analysis of cell morphology, DNA gel electrophoresis) flow cytometry is rapid, objective, and very sensitive. As mentioned, however, since identification of apoptotic cells by flow cytometry is generally based on a single feature, which may not necessarily be the marker of apoptosis in every situation, the mode of cell death should be confirmed by light or electron microscopy. A variety of flow cytometric methods currently exist to identify apoptotic cells, as reviewed above. New methods are continuously being developed and proposed. Unfortunately, many new approaches are just technical modifications of earlier methods, and do not offer additional advantages or novel applicabilities. The multiplicity of these often redundant methodologies may confuse new researchers entering the field rather than helping them to select the most optimal approach. It should be the editorial policy of methodology journals to accept papers describing new methods only if an advantage of these methods over the existing ones is demonstrated.

Flow cytometric methods to quantify apoptotic cells have already found application in clinical oncology. Tumor apoptotic index prior to treatment appears to have predictive value, at least in some tumor types (e.g., 50). Clearly, the knowledge of the rate of cell proliferation and cell death is more predictive of the rate of tumor growth than information on the rate of cell proliferation alone. Furthermore, analyzing the rate of death of tumor cells during treatment (Fig. 8) offers a unique possibility to assess the efficiency of the treatment very early, before other clinical parameters of treatment efficiency can be

measured. Such an application revealed the kinetics of cell death in various types of leukemia treated with different drugs (40,60,89). There is no adequate evidence yet to conclude whether the assessment of the apoptotic index during treatment has long-term prognostic value, but with some drugs, (e.g., the DNA topoisomerase I inhibitor topotecan or microtubule poison taxol) apoptosis appears to correlate well with the initial clinical response (89). Another clinical application of these methods where apoptosis also appears to be of predictive value is in the analysis of spontaneous—or activation induced—apoptosis of lymphocytes in the course of HIV infection (68). These routine clinical applications call for development of standardized procedures and for quality control that could be generally accepted in clinical practice.

The second area of cell necrobiology where flow cytometry is already widely applied is in the study of molecular mechanisms associated with cell death. In this area, flow cytometry offers unique opportunities and is even more advantageous compared to alternative techniques. By virtue of the possibility of correlating measurements of multiple parameters on the same cells, the methodology allows one to detect cells undergoing apoptosis with respect to their cell cycle position, without the need for cell synchronization, or to study the relationship between apoptosis and a particular function of the cell or cellular organelle. Multiparameter analysis combining immunocytochemical detection of individual proteins with apoptotic markers and with DNA content analysis is a powerful approach which can be used to relate expression of individual genes to cell cycle position and the cell's propensity to undergo apoptosis. This allows one to study interactions between components of the regulatory machinery of the cell cycle and apoptosis, mechanism of action of cytotoxic drugs, and the effects of biological modifiers on target cells and other molecular interactions associated with cell death. Needless to say, the molecules directly related to the regulatory mechanisms of apoptosis, such as proteins of the *bcl2* and ICE families, the status of *c-myc* or *ras* proteins, or the expression of the tumor suppressor p53, can be directly measured and correlated with each other, or with still other cell components related to cell death, proliferation or differentiation. One may expect that flow cytometry will contribute to the progress in cell necrobiology as much, or even more, as it did to the progress of research on cell proliferation and the cell cycle.

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