Cytometric analyses to distinguish death processes

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Abstract
The morphological changes typical of apoptosis, as well as the loss of integrity of the plasma membrane and the breakdown of nuclear DNA provide numerous features that permit recognition of apoptotic cell death by various methods. Flow cytometry (FCM) and laser scanning cytometry (LSC) allow for accurate and rapid measurement of apoptosis in both cultures and clinical samples (e.g. solid tumors, bone marrow aspirates, peripheral blood etc.). Furthermore, both FCM and LSC enable one to correlate the apoptosis with the position of the dying cell in the cell cycle. Discussion includes the cytometric identification and quantitation of apoptotic or necrotic cells, based on the analysis of a particular biochemical or molecular feature that is characteristic for either necrosis or apoptosis.

Introduction
Cell death can be divided into oncosis and apoptosis (Majno & Joris 1995, Darzynkiewicz et al. 1997). Both of them eventually lead into oncotic or apoptotic necrosis. Apoptosis occurs in untreated tumors as a consequence of many factors, e.g. deprivation of oxygen and other nutrients or individual cell targeting by hunter-killer cells like macrophages or NK cells (Bowen et al. 1998). The tumor cells die by apoptosis during chemotherapy or radiotherapy and monitoring the level of apoptosis ('apoptotic index') may prove useful in modulating treatment or in predicting the biological behavior of tumors following treatment (Gorczyca et al. 1993a, Fisher 1994). In malignant neoplasms, the rate at which a tumor grows depends on the cell proliferation and cell death (Wyllie 1992, Bowen et al. 1998). Although these two processes appear to be opposing each other, they are linked as is evident from light microscopical observation of malignant tumors. Usually, the more aggressive tumors with high mitotic rates are also noted to have a much more pronounced degree of cell death (see Dowsett et al. 1999 and Lipponen et al. 1999, both in this issue). On the molecular level, the relationship between proliferation and cell death can be explained by the participation of the same oncogenes in the regulation of those processes: oncogenes which force cells into cell cycle by omitting cell cycle checkpoints also induce apoptosis or ‘mitotic catastrophe’.

Apoptosis is regulated by products of many oncogenes or tumor suppressor genes, including c-myc, wt p53, and the family of bcl-2 and caspases (Arends et al. 1990, Arends & Wyllie 1991, Wyllie 1992, Vaux 1993, Bowen et al. 1998) which, together with the role of apoptosis in tumor progression, explain the wide interest in apoptosis in recent years. Because of this interest, numerous techniques have been developed to study apoptosis and to differentiate it from oncosis (necrosis) (see reviews by Darzynkiewicz et al. 1992, 1997). The morphological criteria on which recognition of apoptosis is based still remain the gold standard of analysis of this mode of cell death. The characteristic morphological features of apoptosis are: reduced size due to cell shrinkage and cytoplasmic condensation, plasma membrane undulations ('blebbing'), condensation of chromatin beginning at the periphery of the nucleus followed by nuclear fragmentation (karyorrhexis), dilatation of endoplasmic reticulum and, finally, formation of apoptotic bodies (Arends et al. 1990, Arends & Wyllie 1991). The cell organelles remain relatively unchanged in the early stages of apoptosis. The events described above usually occur within 30-60 min. Necrotic cells (oncosis) are characterized by cell and nuclear swelling, patchy chromatin condensation, swelling of mitochondria, vacuolization of cytoplasm and plasma membrane rupture leading to the formation of ‘ghost-like’ cells and, finally, dissolution of DNA (karyolysis).

Cytometric detection of apoptosis
The morphological changes typical of apoptosis, loss of integrity of the plasma membrane and breakdown of nuclear DNA provide several features that permit recognition of apoptotic cell death by a number of
methods (Darzynkiewicz et al. 1992, Gorczyca et al. 1997).

Flow cytometric identification and quantitation of apoptotic or necrotic cells are generally based on the analysis of a particular biochemical or molecular feature that is characteristic for either necrosis or apoptosis (Darzynkiewicz et al. 1992, Dive et al. 1992, Elstein & Zucker 1994, Steck et al. 1996, Gorczyca et al. 1998, Gorczyca et al. 1994). The most commonly applied flow cytometric methods are based on detection of endonucleolytic DNA degradation that results in extraction of low molecular weight DNA from the cell; such cells are then recognized by their fractional DNA content (sub-G1, peak, sub-diploid peak). When DNA extraction is prevented by crosslinking via cell fixation with formaldehyde, the in vivo presence of numerous DNA strand breaks in apoptotic cells can be detected by labeling their 3' OH termini with fluorochrome-conjugated nucleotides in a reaction utilizing exogenous terminal deoxynucleotidyl transferase (TdT assay, 'TUNEL', in situ end-labeling) (Gorczyca et al. 1992, 1993b). TdT assay can use digoxigenin-biotin or directly fluorochrome-conjugated nucleotides, or can be based on incorporation of BrdUr and its subsequent detection by FITC-conjugated anti-BrdUr antibody (Darzynkiewicz et al. 1997).

Other methods are based on detection of apoptosis-associated changes in distribution of plasma membrane phospholipids or transport function of the membrane. In live non-apoptotic cells, the plasma membrane phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane, and phosphatidylserine (PS) is almost exclusively observed on the inner surface of the membrane. Early in apoptosis there is a breakdown of this asymmetry and PS undergoes translocation to the external leaflet of the plasma membrane. Annexin V, a Ca2+-dependent anticoagulant protein, has high affinity for negatively charged PS and, when conjugated with a fluorochrome (e.g. Annexin V-FITC) can be used as a marker to identify apoptosis (together with DNA staining with propidium iodide, PI). Non-apoptotic cells are Annexin V-negative and PI-negative (negative green and red fluorescence), early apoptotic cells are Annexin V-positive but PI-negative, and late apoptotic cells as well as necrotic cells are stained intensely with PI (only red fluorescence). The Annexin V assay offers the possibility of detecting early phases of apoptosis before the loss of cell membrane integrity and permits measurement of the kinetics of apoptotic death in relation to the cell cycle. It is a very easy and fast method and can be applied in the analysis of apoptosis in cell cultures or leukemic cells from peripheral blood or bone marrow aspirates. It is not, however, very useful in evaluation of apoptosis in solid tumors, most likely due to damage to the membrane during processing and/or disintegration of the tumor when preparing samples for flow cytometry.

Several fluorochromes can serve as markers of decreased mitochondrial transmembrane potential (∆Ψm), which can be used in cytometric discrimination of viable versus dying cells. The most commonly used dyes are Rhodamine 123 (Rh123, green fluorescence) and 3,3' dihexiloxadicarbocyanine (DiOC6, green fluorescence). The mitochondrial changes are among the earliest changes in apoptosis. When Rh123 or DiOC6 is applied together with PI (red fluorescence), live cells stain with Rh123 or DiOC6 (green fluorescence, no PI staining because of intact membrane function). Cells with compromised plasma membrane (necrotic and late apoptotic cells) stain only with PI, showing red fluorescence. The early apoptotic cells in contrast to viable cells will have markedly diminished stainability with Rh123 or DiOC6 and will have dim green fluorescence. JC-1 is another probe which can serve to measure ∆Ψm. Its binding to mitochondria is detected by the shift in color of fluorescence from green, which is characteristic of its monomeric form, to orange which reflects its aggregation in mitochondria, driven by the transmembrane potential. During apoptosis there is marked decrease in orange, and to a lesser degree in green fluorescence of JC-1. One has to keep in mind, however, that the uptake of these cationic probes mentioned above depends not only on the mitochondrial transmembrane potential, but also on transport of these molecules through the plasma membrane. In multidrug resistant cells, these fluorochromes can be pumped out and their measurement will not reflect the exact status of the cell. The apoptosis-associated changes in cell size and granularity can be detected by analysis of laser light scattered by the cell in forward or side directions. Light scatter changes, however, are not characteristic for apoptosis and this method cannot be used to analyze apoptosis in heterogeneous cell populations.

The major advantage of flow cytometry is that, like no other methodology, it offers the possibility of multi-parametric analysis of several cell attributes, including cell cycle position. On the other hand, the major drawback of flow cytometric methods stems from the fact that identification of apoptotic or necrotic cells is not based on morphology and cannot be correlated with morphological classification. Rather, it relies on a single parameter reflecting the change in biochemical or molecular attributes of the cell, presumed to represent either apoptosis or necrosis. Most of the flow cytometric methods do not allow for a clear distinction between late apoptotic and necrotic cells. When in doubt, extensive DNA fragmentation detected by DNA gel electrophoresis or TdT assay may serve as a marker of apoptosis rather than necrosis. Flow cytometric measurement of apoptosis,
regardless of the method used, is associated with selective loss of apoptotic cells during sample preparation.

Laser scanning cytometry (LSC) is a microscope-based cytofluorimeter that combines the advantages of flow cytometry and image analysis (Kamentsky & Kamentsky 1991). Fluorescence of individual cells is measured rapidly, with high sensitivity and accuracy comparable to that of flow cytometry. Since the cells are prepared and measured on a slide, LSC is especially useful when clinical material is limited, as in fine needle aspirates or touch smears. The major advantage of LSC, however, is that each cell’s coordinates (X-Y position) are recorded together with fluorescence values, so that any selected cell(s) can be relocated for visual examination (light or fluorescence microscope) or to capture its image by CCD camera (‘Compusorting’). Almost all of the methods developed for identification of apoptotic and necrotic cells can be modified and adapted for LSC. LSC measurement of total nuclear or cellular fluorescence is done by integration of light intensity of individual pixels over the area of nucleus and/or cytoplasm, measuring both the intensity of individual pixels as well as the fluorescence area (number of pixels). Maximal pixel intensity within the measured area is also measured. Because apoptotic cells, similarly to mitotic cells, have strongly condensed chromatin, they can be identified based on values of the maximal pixels of DNA-associated fluorescence. This approach, however, cannot differentiate between apoptotic and mitotic cells.

The choice of method used to study apoptosis depends on many factors, including type of specimen (cell cultures versus solid tumors), the stage of cell death (early versus late apoptosis) and the technical possibilities available to the laboratory.

References

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