Flow Cytometry in the Study of Cell Death

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In this report we present a concise review concerning the use of flow cytometric methods to characterize and differentiate between two different mechanisms of cell death, apoptosis and necrosis. The applications of these techniques to clinical and basic research are also considered. The following cell features are useful to characterize the mode of cell death: (1) activation of an endonuclease in apoptotic cells results in extraction of the low molecular weight DNA following cell permeabilization, which, in turn, leads to their decreased stainability with DNA-specific fluorochromes. Measurements of DNA content make it possible to identify apoptotic cells and to recognize the cell cycle phase specificity of apoptotic process; (2) plasma membrane integrity, which is lost in necrotic but not in apoptotic cells; (3) the decrease in forward light scatter, paralleled either by no change or an increase in side scatter, represent early changes during apoptosis. The data presented indicate that flow cytometry can be applied to basic research of the molecular and biochemical mechanisms of apoptosis, as well as in the clinical situations, where the ability to monitor early signs of apoptosis in some systems may be predictive for the outcome of some treatment protocols.

Key words: apoptosis - flow cytometry - cell death - necrosis

The two modes of cell death, apoptosis and accidental cell death (necrosis), differ fundamentally in their morphology, biochemistry and biological relevance (Majno & Joris 1995). Depending on the cell type and the stimulus, a cell may die by either of these two distinct ways. Necrosis, a nonspecific mode of cell death, is characterized by swelling of the cell and the mitochondria, which results in disruption of the cell membrane and in lysis (Schrek et al. 1980, Kerr & Harmon 1991). Release of the cytoplasmic content leads to an inflammatory response.

Apoptosis appears to be an active process that plays a role in tissue and organ development (Lockshin & Zakeri 1991), regulation of immune responses (Duke 1991, Abbas et al. 1997, Cohen 1999), or natural death of differentiated cells at the end of their life span (Lockshin & Zakeri 1991). Accurate identification and quantification of the mode of cell death occurring in a particular situation is a necessary prerequisite to an understanding of the biological process taking place. Apoptosis can be defined by morphological criteria, including chromatin condensation and margination, cell shrinkage, membrane blebbing, and formation of apoptotic bodies (Darzynkiewicz et al. 1992, Dive et al. 1992). Apoptosis is also characterized biochemically by enzymatic internucleosomal DNA destruction (Catchpoole & Stewart 1995, Darzynkiewicz et al. 1997). Unfortunately, both morphological and biochemical evaluation of apoptosis lack accurate quantification and sensitivity. While cell death identification has exclusively relied upon light or electron microscopy (Walker et al. 1988, Fesq et al. 1994), methods used for quantitation have included microscopy (Searle et al. 1982), colony-formation assays (Pollack & Ciancio 1989), vital dye exclusion tests (Hudson & Hay 1986) and flow cytometry (Nicoletti et al. 1991, Darzynkiewicz et al. 1992, 1997, Schmid et al. 1994a,b, Vermes et al. 1995, Douglas et al. 1998).

The capacity of flow cytometry for rapid, individual analysis of a large number of cells would appear to make it ideally adapted for the study of cell death. Several flow cytometry methods are available that primarily use alterations in DNA, light scatter properties, or surface membrane to assess cell death (Koopman et al. 1994, Schmid et al. 1994b, Darzynkiewicz et al. 1997, Ferlini et al. 1997). Thus, apoptotic cells can be recognized by their diminished stainability with DNA specific fluorochromes such as propidium iodide (PI) (Fig. 1), DAPI, acridine orange, or Hoechst dyes, due to DNA degradation and its subsequent leakage from the cell. In contrast to apopotic cells, necrotic cells generally do not show an immediate reduction in DNA stainability. Thus, the discrimination between normal live or necrotic cells is impossible based

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on single-parameter DNA content analysis alone. Changes in the morphology of cells undergoing apoptosis affect their light scattering properties. The decrease in forward light scatter can be observed in thymocytes triggered to undergo apoptosis, paralleled by an increase in side scatter. Cell death by necrosis results in a increase of the forward and side scatter signals. The integrity of the plasma membrane of cells undergoing apoptosis is preserved and most functions of the membrane remain unchanged (Arends et al. 1990). Apoptotic cells, thus, exclude "viability assay" dyes such as trypan blue or PI. This is in contrast to necrotic cells, in which one of the earliest changes is the loss of membrane function and its structural integrity. By their ability to exclude PI, apoptotic cells can be erroneously classified by flow cytometry as viable cells. To evaluate apoptosis in a mixed cell population, identification of cell phenotype is necessary. Several currently available techniques allow phenotypic discrimination of cell populations and simultaneous quantification of apoptosis (Telford et al. 1992, Schmid et al. 1994a, Sherwood & Schimke 1995, Chiu et al. 1996). An assay of cell viability based on simultaneous cell staining with PI and HOECHST 33342 (HO342) provides a means to discriminate between live vs apoptotic vs necrotic cells (Pollack & Ciancio 1991). PI has been the stain most often used to determine apoptotic events, however it produces staining overlap with PE. Additionally, the cell membrane must be permeabilized and therefore can not be stained for surface markers. Some methods use HO342 for measurement of DNA content or apoptosis, however, this dye requires UV excitation which hinders its general use. Furthermore, PI and HO 342 can compromise the simultaneous assessment of cell cycle or aneuploidy. Measurements of cell cycle or aneuploidy can be vital for investigations in tumor systems. In this context, Douglas et al. (1998) described a new approach for simultaneous evaluation of cell phenotype, apoptosis and cell cycle using the TUNEL method (previously described by Gavrieli et al. 1992) to determine apoptosis, while cell cycle information was assessed with an ultraviolet DNA binding dye, DAPI. To simultaneously determine surface phenotype they combined with FITC- and PE-labeled surface antibodies. Nevertheless the use of three-laser instrumentation prevents its customary use. Another assay which combines the staining with annexin V and PI has been used to discriminate early apoptotic cells from late apoptotic and necrotic ones, based on the determination of the translocation of phosphatidyl serine from the inner to the outer layer of the plasma membrane of early apoptotic cells, stained by annexin V (Vermes et al. 1995).

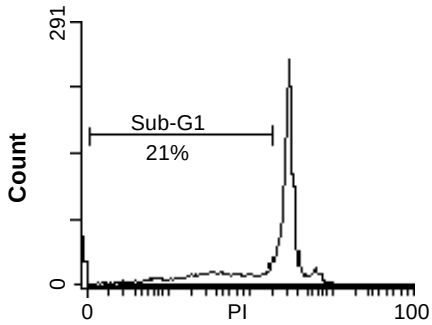

Fig. 1: analysis of apoptosis by hypotonic PI staining method. In this experiment cells were cultured in the presence of Staurosporine for three days to induce early apoptosis and then stained by the hypotonic PI assay. The sub-G1 region encompasses the apoptotic cells.

Inaccurate apoptosis determinations can be readily overcome by simultaneous cell surface staining of apoptotic cells. The acridine orange/ethidium bromide (AO/EB) assay cannot be used for phenotypic analyses due to broad emission spectrum of AO and EB. The *in situ* nick translation (ISNT) assay permits phenotypic measurements on apoptotic cells (Meyaard et al. 1994), but we ascertained that an underestimation of apoptosis could occur in some subsets due to a decrease in cell surface antigen expression associated with apoptosis (Swat et al. 1991, Schmid et al. 1994a,b) and ethanol fixation (Lecoeur & Gougeon 1996). In fact, the more reliable method to identify the cell subsets involved in the apoptosis process is the combination of 7-AAD staining with FITC or PE cell surface staining (Schmid et al. 1992, 1994 a,b). Nevertheless, Lecoeur and Gougeon (1996) have showed that erythrocytes can significantly interfere in the 7-AAD assay because of their size which is similar to lymphocytes. However this interference could be easily avoided by targeting the apoptotic cells with appropriated cell surface monoclonal antibodies, such as CD45.

APPLICATIONS IN HUMAN DISEASES

Regarding apoptosis, it has been hypothesized that this phenomenon may represents an important modulatory mechanism. In this connection, several studies in Aids patients have suggested that apoptotic events occurring in $CD4^+$ and/or $CD8^+$ T lymphocytes may aggravate the progression of the disease (Gougeon et al. 1993, 1996, Ameisen et al. 1995, Echaniz et al. 1995, Neves Jr. et al. 1998). Several viruses are known to induce apoptosis, as exemplified by infection of human peripheral monocytes with dengue-1 and dengue-2 viruses (von Sydow et al. 1998).

Several flow cytometric techniques have been used on the studies about the occurrence of cell death in protozoan diseases (reviewed by Barcinski & DosReis 1999). Some works have also focused on its possible role in the aggravation of experimental Chagas disease (Lopes & DosReis 1995, 1996). Activation-induced cell death of $CD4^+$ T cells from *Trypanosoma cruzi* infected mice abrogate IFN-γ production and up-regulates parasite replication in macrophages *in vitro* (Nunes et al. 1998). In leishmaniasis there is evidence suggesting that the sensitivity of *Leishmania* promastigotes to programmed cell death (PCD) is induced by heat shock and modulated by calcium (Moreira et al. 1996). It has also been demonstrated that the intracellular infection by *L. donovani* inhibited PCD in macrophages (Moore & Matlashewski 1994).

Human localized cutaneous leishmaniasis (LCL) induced by *L. braziliensis*, ranges from a clinically mild, self-healing disease with localized cutaneous lesion to severe forms, which can involve secondary metastatic lesions. T-cell-mediated immune response is extremely important to define the outcome of the disease; however, the underlying mechanisms involved are not fully understood. We adapted a 7-AAD assay in combination with $CD4^+$ or $CD8^+$ T cell surface phenotyping to verify whether different levels of early apoptosis or late apoptosis and accidental cell death were found at different stages of LCL lesions (Fig. 2). When cells, separated from a biopsy sample and displaying normal viability patterns, were gated, higher levels of early apoptotic and dead cells were observed in lesions from patients with active disease as compared with lesions on spontaneous healing. Hence, higher levels of early apoptotic cells were detected among $CD8^+$ T cells as compared with $CD4^+$ T cells from patients with active disease. These data suggest that higher proportions of cell death events in $CD8^+$ T cells from patients with LCL would be associated with active disease. Interestingly, it was observed low amounts of early apoptotic events among $CD8^+$ T cells derived from patients with self healing lesions (Fig. 3). It is possible to speculate that, during the immune response, differences in apoptotic events in $CD4^+$ and $CD8^+$ T cell subsets could be responsible for changing the CD4/CD8 balance, thus leading to healing or maintenance of disease (Bertho et al. 2000).

THE FUTURE

It seems probable that the future applications of flow cytometry to studies of apoptosis could develop in three ways. The first direction would

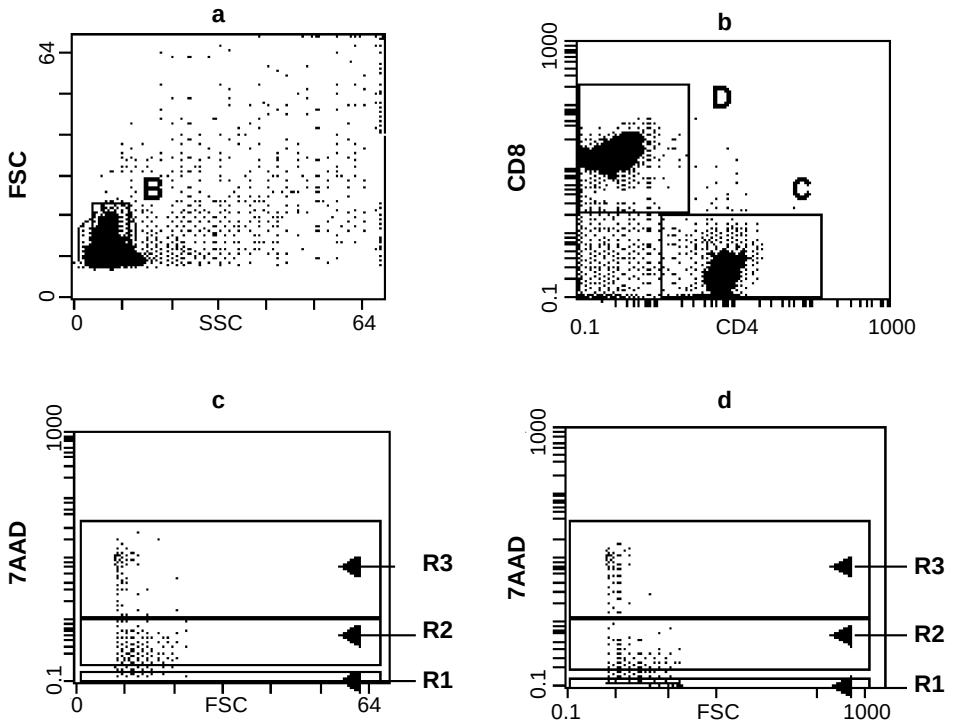

Fig. 2a: representative flow cytometric analysis to determine live (R1), early apoptotic (R2) or dead (ACD) (R3) $CD8^+$ or $CD4^+$ T cells, obtained from lesion of localized cutaneous leishmaniasis patient. a: scatter histogram (SSC/FSC) in which gate B were created, following viability scatter patterns; b: dual-color dot plot of $CD8^+$-PE and $CD4^+$-FITC T cells based on gate B; c: FSC vs. 7-AAD gated on $CD8^+$ T cells within gate D; d: FSC vs. 7-AAD gated on $CD4^+$ T cells within gate C.

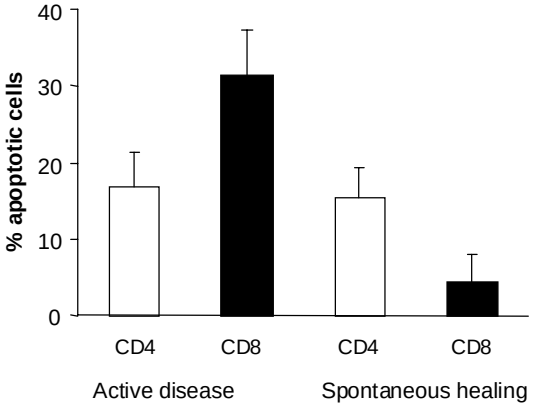

Fig. 3: early apoptotic in $CD4^+$ or $CD8^+$ T cells obtained from lesions of patients with active disease or spontaneous healing as measured by flow cytometry. Solid bars represent early apoptotic $CD8^+$ T cells, and open bars, early apoptotic $CD4^+$ T cells. ($P = 0.0001$); ($P = 0.0004$).

be in the field of cellular and molecular biology and pharmacology. It would involve further investigation of various metabolic features of cells undergoing apoptosis, to better comprehend the mechanisms of this process. A diversity of markers can be used to reveal molecular changes that occur in the sequence of events that lead to DNA degradation and cell death. The second area in which flow cytometry would prove useful is more related to the clinic. This methodology offers promise for the early detection of apoptotic cells in tumors during treatment. Sampling of tumors for apoptotic cells may offer a very early marker of tumor response, predictive of cancer's sensitivity to a given treatment. The third application is to immunological studies concerning the pathogenesis of a variety of human diseases including protozooses (such as Chagas disease, leishmaniasis, toxoplasmosis and leprosis), cancer, autoimmune diseases, neurodegenerative disorders, Aids and viral infections, such as dengue. There are many aspects which should be investigated to better understand the mechanisms involved in the pathogenesis of these diseases.

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