Assessing apoptosis: a critical survey

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Abstract
Much progress has been made in the understanding of the process of apoptosis and there is a burgeoning literature pointing to it being a key phenomenon in tissue homeostasis. Moreover it is clear that derangements of the apoptotic cascade and its regulation can occur in a variety of disease states including in cancer. Many methods exist for the demonstration of apoptosis but some are not quantifiable. Others, such as enumeration of apoptotic bodies or end-labelling techniques, lend themselves to quantification. However, poor methods of quantification are sometimes employed. In this review, the methodological problems that can affect the assessment of apoptosis are discussed and suggestions made for more rigorous approaches.

Introduction
The notion that apoptosis represents a critical element in cell number control in physiological and pathological situations has been well reviewed and its role in oncogenesis is now well established (White 1996, Ashkenazi & Dixit 1998, Evan & Littlewood 1998, Thornberry & Lazebnik 1998, Green & Reed 1998). Furthermore, there is increasing recognition that many of the effects of chemo- and radio-therapeutic agents are mediated by apoptosis (Eastman 1990, Dive & Hickman 1991, Schmitt & Lowe 1998). The seminal work of Kerr, Wyllie & Currie (1972), building upon the earlier observations of Glucksmann (1951) and Saunders (1966), should be read by those interested in assaying apoptosis because of the excellent photomicrographs that document the morphological features of the process. This is important since despite considerable progress in the understanding of the mechanistic basis of apoptosis, morphological analysis remains unquestionably the ‘gold-standard’ for its assessment and quantitation.

Apoptosis is a regulated and active process and the regulatory processes involved are being increasingly understood (Schmitt & Lowe 1999, Ashkenazi & Dixit 1998, Evan & Littlewood 1998, Thornberry & Lazebnik 1998, Green & Reed 1998). Whilst a diverse range of insults and physiological events can lead to apoptosis the process is remarkably stereotyped, with a programme of activities leading to the final morphological events which are similar throughout phylogeny and may be recapitulated in most (if not all) cell types. Mounting data indicate that much of the machinery for the implementation of the apoptotic response is ‘hard-wired’ in cells, being present all the time but kept in an ‘off’ state: and rapidly recruited into an ‘on’ state if needed. Consequently, and despite much effort, there remain few biochemical markers of the apoptotic process that are specific for this complex regulated process. Similarly, while many potential regulators of apoptosis are described, critical examination of the available data indicates that there is little consensus on their value as markers of apoptosis.

A critical point for the quantitation of apoptosis is that, irrespective of the initiating insult, the time course of apoptosis is very fast (Sanderson 1976, Matter 1979). Moreover, the clearance of the resultant debris (either by ‘professional’ phagocytes or bystander (amateur) phagocytes) is rapid. Very few data are available to substantiate this widely held idea but Coles et al. (1993) suggested that clearance times of less than 1 h were typical. The rapid nature of apoptosis means that in any static analysis, a very small number of apoptotic cells observed at a given instant might, in fact, reflect a very considerable contribution to cell turnover. Dramatic evidence for this came from studies of the physiological contribution of apoptosis in renal development (Coles et al. 1993) and in the steady-state regulation of intestinal epithelial populations (Hall et al. 1994). While the process of apoptosis and its clearance is (as far as we can tell) always rapid there may be some variation between cell types or in relation to different insults. This may have important implications for the quantitation of apoptosis (Potten 1996).

Given its contribution to cell turnover in physiological, pathological and toxicological situations, it is important to be able to identify and quantitate the process of apoptosis in cells and in tissues. Ideally, one would like a technique

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Given its contribution to cell turnover in physiological, pathological and toxicological situations, it is important to be able to identify and quantitate the process of apoptosis in cells and in tissues. Ideally, one would like a technique
which is sensitive and highly selective for apoptosis, and it should also be easily applicable to routinely prepared tissue sections. An important issue is the clear distinction between rate and state measurements (Hall & Levison 1990, Hall & Coates 1995). This is illustrated by the following analogy. Standing on a bridge overlooking a motorway there may be at a given instant 50 cars below you. This is a state parameter and tells you nothing about whether they are stuck in a traffic jam or moving at 100 miles per hour! The latter involves a time dependent variable, hard to measure in most situations! The measurements made of apoptosis are almost invariably state parameters: it is quite wrong therefore to talk of apoptotic rates!

The measurement of apoptosis

There are a number of methods for measuring apoptosis. The phenomenon of apoptosis is defined by a series of morphological changes (Kerr et al. 1972, Wyllie et al. 1980, Kerr et al. 1987). The classical features are best seen by electron microscopy but can be observed at the light microscopic level using nucleic acid-binding dyes, such as haematoyxlin, acridine orange, or propidium iodide (Coles et al. 1993, Hall et al. 1994). The first signs of apoptotic cell death are a condensation of the nuclear material, with a marked accumulation of densely stained chromatin, typically at the edge of the nucleus. This is accompanied by cell shrinkage. Cytoplasmic blebs appear on the cell surface, best seen by time-lapse video-microscopy (Evan et al. 1992), and the cell detaches from its neighbours. The nuclear outline often becomes highly folded and the nucleus breaks up, with discrete fragments dispersing throughout the cytoplasm. Eventually, the cells themselves fragment, with the formation of a number of membrane bounded apoptotic bodies. Apoptotic bodies are generally phagocytosed by surrounding cells, which are not necessarily derived from the mononuclear phagocytic system. Therefore, the most common sign of apoptosis in a tissue section is the presence of apoptotic bodies, which may be seen as extracellular bodies, or, after phagocytosis, inside other cells. Apoptotic bodies have a diverse appearance, particularly in regard to their size. They are generally oval or round in shape, and are most easily recognised when they contain large amounts of homogeneous, condensed chromatin. The morphological features of apoptosis have been extensively reviewed, and plentiful illustrations of both the light and electron microscopic appearances are provided in these publications (Kerr et al. 1972, Wyllie et al. 1980, Ucker 1991).

Although historically, morphological methods (including ultra-structural methods) have been favoured, the recognition that apoptosis was associated with DNA fragmentation with the cleavage of DNA between nucleosomes has been regarded as a key parameter. Unfortunately both this and other DNA based methods involving electrophoresis not only lose critical spatial information but also are not easily quantified. Similarly, the extreme sampling error inherent in electron microscopic methods makes quantitation by this technique fraught with difficulties. In the early 1990s several methods which take advantage of the DNA cleavage associated with apoptosis but applicable to cytological and histological material were popularised (Gavrielli et al. 1992, Ansari et al. 1993, Gold et al. 1993, Wijsman et al. 1993). These certainly have utility and can be used to identify apoptotic bodies in tissues and cells. While not without problems (for example in relation to fixation and sample processing (Ansari et al. 1993, Coates et al. 1995, Save et al. 1998)) these methods are often useful, particularly for those not experienced in microscopy (Save et al. 1998).

The property of DNA fragmentation in apoptosis (Wyllie 1980, Wyllie et al. 1984) can be utilised to identify cells undergoing this process (Gavrielli et al. 1992, Ansari et al. 1993, Wijsman et al. 1993, Gold et al. 1993) since certain enzymes can add labelled nucleotides to the DNA ends. The labelled nucleotides can then be identified by immunological methods akin to immunohistochemistry. Such methods were originally termed TUNEL (terminal deoxynucleotidyl transferase mediated UTP nick end labeling) but are also referred to as ISEL (in situ end-labeling techniques). Strictly, the different names relate to the different enzymes employed and while there are theoretical and some practical differences, the similarity of technique and result make the names essentially interchangeable. A comparison of the methodologies employing TdT (TUNEL) and Klenow fragment of DNA polymerase (ISEL) was reported by Mundle et al. (1995) who demonstrated that TUNEL appeared more sensitive than ISEL. This is because TdT can label 3' recessed, 5' recessed or blunt ends of DNA, whereas ISEL labels only those with 3' recessed ends. All three types of DNA end are seen in apoptosis and thus in principle TdT based methods should be more sensitive than Klenow polymerase methods. Despite this in practice ISEL and TUNEL appear functionally interchangeable (Save et al. 1998).

Irrespective of the enzyme employed a variety of labels could be used, including radioactive nucleotide triphosphates. However, methods based on the use of non-isotopic labels have been developed and are superior for a variety of reasons, including ease of use, stability, simplicity and speed of detection, and the increased resolution obtained. Using this approach, it has been clearly shown that the amount and distribution of labeled cells is closely correlated with the amount and distribution
of cells known to be undergoing apoptosis using other methods (Ansari et al. 1993). The method can be modified for fluorescence detection in situ or by flow cytometry or detection at the light or ultrastructural levels (Gold et al. 1993, Migheli et al. 1995). In addition, the use of immunocytochemistry for cell surface antigens in combination with TUNEL/ISEL allows the identification of the particular cell types undergoing apoptosis, and could also be used to measure phenotypic changes in apoptotic cells (Gold et al. 1993, Kurrer et al. 1997) and with in vivo hybridisation methods (Strater et al. 1996).

In recent years, a large number of antibodies have been marketed for apoptosis research. The vast majority of these recognise proteins which can influence apoptosis in certain instances, but are neither universal nor specific. Indeed, since apoptosis is a consequence of the activation of pre-existing mechanisms within a cell there has been relatively little progress in the identification and use of antigens whose expression is correlated with this form of cell death. Immunohistological detection of the expression of regulatory proteins (eg. Bcl-2 or BAX) has been reported, but the utility of this approach as a marker of apoptosis is unsubstantiated. Moreover, there have been significant problems with the specificity of some reagents purported to be apoptosis specific and the authors urge extreme caution (see for example the caveat on BAX). Unfortunately this method requires the use of unfixed cells and cannot be applied (at present) to histological material. The use of antibodies to clussterin (also known as TRPM-2, or SGP-2) has also been correlated with apoptosis in certain situations, although this protein is not a universal marker, nor is it specific for apoptosis (Garden et al. 1991). Perhaps the most widely used immunohistochemical marker of apoptosis is the identification of tissue transglutaminase (TG) (Garden et al. 1991, Fesus et al. 1989, Cummings 1996), although even here recent data suggests that trans-glutaminase is not always induced during apoptosis (Szondy et al. 1997). Finally, apoptosis can also be demonstrated by in situ hybridisation (Hilton et al. 1997) although the utility of this method also remains to be established.

Quantitation of apoptosis

This is the most important problem. Critical examination of the literature pertaining to apoptosis, particularly in association with clinical material and tumours shows that there is often a lack of rigour in the measurement of cell death. This is evident from the very beginning of many papers. The events to be quantitated are rarely sufficiently well defined; the counting method is often biased; the denominator used in the quantitation is almost never shown to be biologically or statistically relevant; an assessment of the homogeneity or heterogeneity of the sample is only infrequently made. These and related problems are frequently compounded by the situation in which the number of events to be assessed (apoptotic bodies or TUNEL/ISEL labelled cells) is very low as a proportion of the total number of cells. Under such conditions it is usually the case that large numbers of events must be assessed to give statistically verifiable data, and this number increases as the degree of heterogeneity of the sample increases. This author regards those papers that utilise semi-quantitative approaches (+, ++, ++++, +++) as being unacceptable. Similarly those studies using arbitrary denominators such as the use of ‘high power fields’ are to be deprecated. This debate has been thoroughly discussed with regard to the quantitation of mitoses (Scully 1976, Hall & Levison 1990). Finally, those studies where there is no serious consideration of ‘reproducibility’ or assessment of heterogeneity are likely to be flawed.

In some situations it may be acceptable or desirable to use some micro-anatomically defined unit as the denominator in quantitation. For example, Hall et al. (1994) in a study of apoptosis in the human gastrointestinal tract employed histologically defined units such as small or large intestinal crypts or regions of the small intestinal villus as meaningful denominators. In other situations some device is needed to define what a useful denominator might be. Here the author recommends the application of the wandering mean technique. In this method an experiment is performed on a small number of examples from the set of samples to be studied. They are examined in some detail in order to define the validity of counting strategies. In a particular case the first field to be assessed is examined and the number of events is quantitated (e.g. apoptotic bodies (carefully defined)) as a proportion of the total cells in the field. This gives a score of apoptotic events, A1, in the relevant denominator population N1. In the second field the same procedure is followed and this gives a score of A2 in relevant denominator population, N2. The procedure is repeated for many fields and the sum of the apoptotic events (Σ A1+A2+ . . . An) is derived after each field (1,2 . . . n). The cumulative mean is derived by dividing the cumulative score of events by the cumulative denominator population (Σ N1+N2+. . . Nn). If these are plotted the mean will be seen to wander and eventually oscillate about a given value and as N increases the oscillation will dampen, asymptotically approaching the correct value (see Fig. 1). This procedure can then define experimentally the number of events to be assessed to produce a given quality of data. Here, clearly, pragmatism is the key word and the question an investigator must ask.
is ‘What error will I accept?’. By performing this exercise for a small series of cases the validity of a counting strategy can be defined. Certainly it is the case that there may be big differences between samples in a series and what is a suitable denominator in one case may be inadequate in another, perhaps because of considerable heterogeneity.

Conclusion
We have a rapidly developing knowledge of the cellular and molecular basis of apoptosis and an increasing recognition of its contribution to both physiological and pathological processes, including neoplasia. However, our ability to objectively quantitate this process remains poorly developed and much of the literature is filled with methodological errors, some of which are so gross as to invalidate the conclusions drawn. If apoptosis is to be usefully and reliably assessed in histological material then there is no escape from meticulous and painstaking microscopy coupled with rigorous and meticulous quantitation. Whether it is the quantitation of morphologically defined events (‘the gold standard’) or of TUNEL/ISEL defined events is in the author’s view a relatively unimportant issue - they will correlate very well - and both have inherent problems. In the end, the choice of method will depend upon the experience of the researcher in histological analysis and microscopy. What is not in dispute is the need for rigour in the quantitation process.

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