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Invited Review

Methods for detecting apoptotic cells in tissues

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Summary. In this review, methods currently available for the detection of cell death in tissues are surveyed, with special reference to techniques that allow the recognition of apoptotic cells *in situ*, either in whole mount specimens or in tissue sections. The techniques considered include: several variations on *in situ* DNA nick-end labelling methods, vital dyes, lysosomal enzyme histochemistry, transglutaminase expression, and immunocytochemical detection of several death-associated antigens. These methods are discussed in relation to their utility in detecting different stages of cell death, and also to their ability to distinguish between apoptotic and necrotic cell death.

Key words: Cell death, Apoptosis, Methods, Review

1. Introduction

With the current substantial interest in the mechanisms of cell death in tissues, has come a battery of methods for the demonstration of dying cells. Most of the recent studies have centred on the detection of cells undergoing apoptosis, an active and often programmed form of cell death, in contrast to necrosis, which is a general cell lysis resulting from direct or indirect membrane damage. The term "programmed cell death" was first introduced by developmental biologists to describe the temporally regulated death of cells during metamorphosis and development (Lockshin and Williams, 1965; Lockshin, 1969; Sanders and Wride, 1995). As the term implies, programmed cell death involves a timed activation of an intrinsic genetic program and the expression of new proteins. It shares this characteristic with apoptosis, a term introduced by pathologists (Kerr et al., 1972; Wyllie, 1992) to describe a form of cell death with well-defined morphological features. Whether or not all examples of programmed cell death occurring during development are also examples of apoptosis is unclear, resulting in some confusion between the two terms and the tendency to use them synonymously. Certainly, not all programmed cell death in embryos is accompanied by the morphological characteristics of apoptosis, and so these two phenomena most probably share some common pathways, but are not equivalent in all respects (Lockshin and Zakeri, 1991; Schwartz et al., 1993).

While apoptotic cells are generally characterised by well-defined morphological features, controlled cleavage of DNA and the expression of new proteins (Compton, 1992; Gerschenson and Rotello, 1992; Kroemer et al., 1995), necrosis is a more general degeneration characterised by an early failure of membrane integrity, mitochondrial deterioration, and uncontrolled DNA breakdown (Kerr and Harmon, 1991; Lockshin and Zakeri, 1991). Although these two forms of cell death may both present similarly shrunken pyknotic nuclei, with condensed chromatin and hyperchromatism, necrosis is a pathological phenomenon, while apoptosis may be either physiological or pathological. However, just as apoptosis is not necessarily synonymous with programmed cell death, apoptosis is not always clearly different from necrosis (Columbano, 1995), because the characteristics of these forms of cell death which are considered diagnostic are not invariably present in all tissues.

The intense research effort currently underway to understand the genetic and biochemical characteristics of apoptosis, and to understand its full significance, has resulted in advances on many different fronts. Elucidation of the genetic (Wyllie, 1995) and molecular (Vaux and Strasser, 1996) mechanisms of apoptosis has lead to the identification of specific markers of this process which can facilitate the development of techniques for the identification of cells undergoing this form of death. For example, the distinct DNA fragmentation that occurs during apoptosis (Bortner et al., 1995) has triggered the refinement of DNA labelling methods for cells at various stages in the apoptotic cycle.

This review will cover some of the methods now available for the identification of cells that are undergoing apoptosis and programmed cell death, with special emphasis on techniques for detecting these cells *in situ*. Also, because programmed cell death is characteristic of developmental systems, particular consideration will be given to examples occurring during morphogenesis.

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2. Morphology

The light microscopical and ultrastructural features of apoptotic cells have been described previously, both frequently and comprehensively (Wyllie et al., 1980; Walker et al., 1988; Clarke, 1990; Falcieri et al., 1994). The most striking changes occur in the nucleus, where the chromatin becomes condensed often into a crescent-shaped mass aggregated onto the inner surface of the nuclear envelope (margination). The nucleus itself may become fragmented. In the cytoplasm, the ground substance also becomes condensed, and the formation and discharge of fluid-filled vacuoles leads to the characteristic shrinkage that accompanies apoptosis ("shrinkage necrosis", Kerr, 1971). Mitochondria retain their integrity until relatively late in the process. Eventually blebbing occurs, and membrane-bound bodies are pinched off from the cytoplasm (apoptotic bodies), which are phagocytosed.

Although some of these features, such as pyknosis, may be shared by both apoptosis and necrosis, the overall picture of these two phenomena is usually distinct (Wyllie, 1981; Kerr and Harmon, 1991). However, among apparently apoptotic cells, these morphological features are not invariably found, and this is particularly true in developmental programmed cell death (Clarke, 1990; Sanders and Wride, 1995). Nevertheless, the value of observing morphological changes cannot be underestimated; it is sometimes necessary, and always desirable, to confirm that the putative apoptotic cell in fact shows at least some of the morphological features of the apoptotic state. Although in the past many studies of developmental cell death have depended exclusively or heavily on morphological criteria (Bellairs, 1961; Pexieder, 1975; Dyche, 1979; Mills and Bellairs, 1989), it is now possible to confirm cell death using a battery of other methods.

3. Light microscope stains and cytochemistry

A wide range of histological and histochemical methods have been used for the demonstration of dying cells in tissues (Bowen, 1981), but only a few of these have been employed routinely in recent studies. Among the more widely used vital stains are nile blue sulphate (Fig. 1) and neutral red. The former has been particularly useful for the demonstration of dying cells in embryos since its introduction by Saunders et al. (1962), and it has been used on many occasions since (Webster and Gross, 1970; Pexieder, 1975; Jeffs and Osmond, 1992). When applied in dilute solution to living tissue, nile blue sulphate and neutral red seem to be concentrated in the dying cells, though by what mechanism is still unclear. Bowen (1981) has suggested this to be due to altered nuclear membrane permeability.

Nile blue sulphate, as well as another vital dye, acridine orange, have been claimed to be specific for apoptotic cell death in *Drosophila* embryos, leaving necrotic cells unstained (Abrams et al., 1993). This conclusion was arrived at by carefully comparing the staining properties of cells with these dyes, with their ultrastructure. Further, these dyes stained cells both before and after their engulfment by phagocytes, indicating that the selective staining of these cells is not merely a consequence of impaired membrane permeability, because the phagocyte membrane was also penetrated by the stains. The authors conclude that the selectivity of nile blue sulphate and acridine orange for apoptotic cells is associated with biochemical changes that are specific to apoptosis.

The study by Abrams et al. (1993) also indicated that toluidine blue staining of fixed and sectioned material was a reasonably reliable marker for apoptotic cells. The distribution of pyknotic nuclei stained by toluidine blue corresponded fairly well with the distribution of acridine orange- and nile blue sulphate-stained cells. A similar result with toluidine blue on fixed tissue was reported by Martín-Partido et al. (1986), who simultaneously stained healthy nuclei in the section with safranin for contrast.

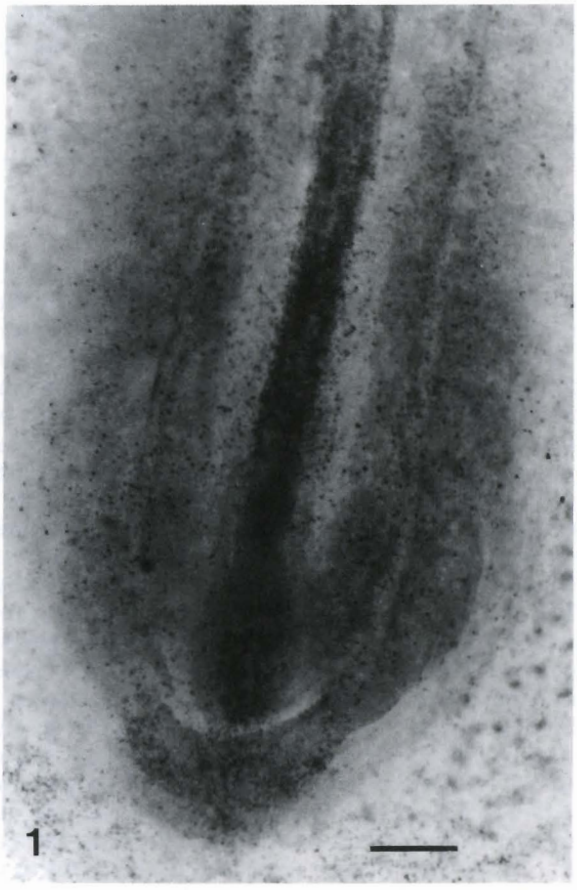

Fig. 1. Whole mount of the tail of a two-day chick embryo, stained with nile blue sulphate. This dye is taken up by many cells in this region of the embryo that are in relatively late stages of cell death. Stained cells are seen scattered throughout the region. Bar: 200 μm.

Trypan blue exclusion viability tests, in which this dye is excluded from living but not from dead cells, rely on compromised plasma membrane permeability during cell death (Bowen, 1981). This method will therefore primarily demonstrate the presence of necrotic cells caused by injury, and will only reveal apoptotic cells when they are relatively far along in the death process.

Acid hydrolase cytochemistry has had a long history of use as a marker of cell death (reviewed extensively by Bowen, 1981). The enzyme most often examined, acid phosphatase, is a well-established indicator of lysosomal activity, however, the acceptability of this approach has declined. The presence of phagocytes in zones of apoptotic cell death (Savill et al., 1993) can make it difficult to decide whether the reaction product is in the phagocytes or the apoptotic cell. Further, it is generally thought that lysosomal integrity is maintained until relatively late in apoptosis, and much of the interest in this phenomenon is centred on early events. Nevertheless, there are reports of apparent lysosomal activity in cells with apoptotic morphology (Poelmann and Vermeij-Keers, 1976; Hurlé and Hinchliffe, 1978), though this may be difficult to separate from autolytic events (Nixon and Cataldo, 1993). These difficulties, together with inconsistencies in results in the literature and the development of new methods, has resulted in the diminished usefulness of this technique for the study of apoptosis.

Among the DNA stains, Feulgen-stained pyknotic nuclei have been used to detect and quantify apoptosis using image analysis (Camby et al., 1995). However, the use of fluorescent general DNA dyes has been far more prevalent (Telford et al., 1992). These include intercalating DNA dyes, such as propidium iodide, ethidium bromide and acridine orange; and externally binding DNA dyes, such as the Hoechst dyes 33342 and 33258, and 4'-6-diaminido-2-phenylindole (DAPI). These dyes have been used primarily in conjunction with flow cytometry (see below), but can be used *in situ*. In the case of ethidium bromide (Lyons et al., 1992), the early stages of apoptosis appear to be associated with faint staining. This conclusion was arrived at after microscopical examination of the morphology of the faintly staining cells, which in this case were murine thymocytes. Early nuclear changes associated with TNF-α-induced apoptosis have also been detected using the intercalating dye *p*-phenylenediamine, although the mechanism is unclear (Salcedo and Fleit, 1992).

The use of the externally DNA-binding Hoechst dyes and DAPI relies on the altered fluorescence of nuclei in which DNA is condensed and fragmented, in comparison with more uniformly stained non-apoptotic cells (Hardin et al., 1992). The mechanism by which this altered binding occurs is unknown, however flow cytometric comparisons show an overall decrease in dye binding by apoptotic nuclei (Telford et al., 1992, 1994). One benefit of these fluorescence techniques is that they can be combined with a second fluorescent label to confirm the phenotype (Hardin et al., 1992; Lyons et al., 1992) or to confirm DNA fragmentation using a nick-end labelling method (Toné et al., 1994).

4. *in situ* labelling of DNA strand breaks

Two approaches have been taken to labelling the fragmented DNA that is characteristic of apoptotic cells. One of these, known as *in situ* nick translation (ISNT) or *in situ* end labelling (ISEL), relies on the incorporation into the fragmented DNA of labelled nucleotides, using DNA polymerase I (Iseki and Mori, 1985; Iseki, 1986; Fehsel et al., 1991; Ansari et al., 1993; Gold et al., 1993; Wijsman et al., 1993). The second approach is a "tailing reaction" which incorporates labelled dUTP into fragmented DNA using terminal deoxynucleotidyl transferase (TdT), and is commonly referred to as TdT-mediated bio-dUTP nick end labelling or TUNEL (Gavrieli et al., 1992). In both techniques the labelled nucleotides are identified histochemically or immunocytochemically. These methods may be applied to both paraffin sections (Fig. 2) and to cultured cells, and comparisons have been made of the two methods with respect to sensitivity and the ability to distinguish between different types of cell death. The latter point is controversial, and both techniques bear the caveat that

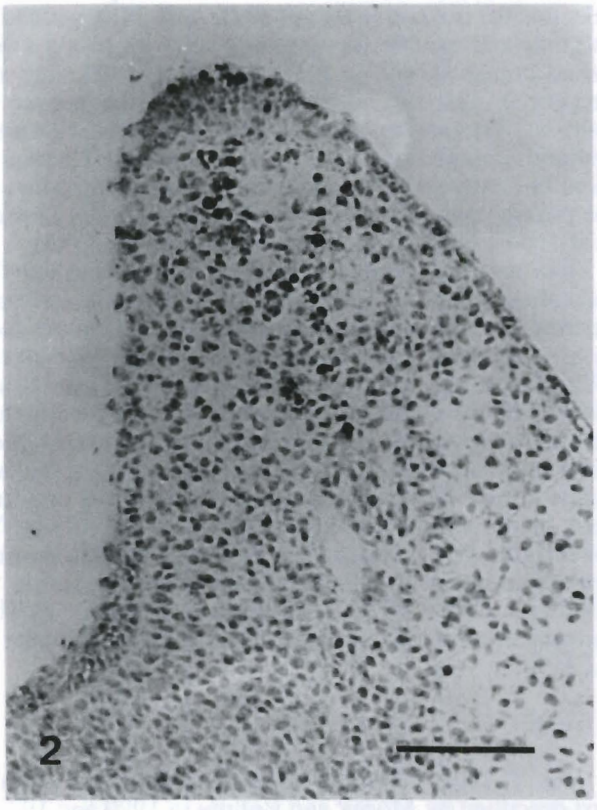

Fig. 2. Section through the anterior limb bud of a four-day chick embryo, labelled for dying cells using the TUNEL technique. The nuclei of cells in the region known to be undergoing programmed cell death are densely labelled. See Wride et al. (1994). Ba: 50 μm.

not all examples of apoptosis may be accompanied by DNA fragmentation (Schulze-Osthoff et al., 1994).

The nick translation methods only label single strand breaks in DNA that expose 3'OH ends, and it needs to be borne in mind that if these termini are blocked they will not be labelled. The use of nuclease digestion has been recommended to overcome this difficulty (Chaudun et al., 1994). The presence of 3' overhangs in the DNA fragments appears to be characteristic of apoptosis, in contrast to necrosis which generates primarily blunt ends to the DNA fragments. This apparent difference may provide a means of distinguishing between these two forms of cell death (Didenko and Hornsby, 1996).

In a related technique, antibodies prepared against single stranded DNA will immunocytochemically label the nuclei of cells undergoing drug-induced apoptosis, and of embryonic cells undergoing programmed cell death (Keino et al., 1994; Naruse et al., 1994). The method presumably relies on the degradation of DNA during apoptosis into single strands as well as into internucleosomal fragments.

a. in situ nick translation

This method can be used to follow the progress of DNA damage at the single cell level (Fehsel et al., 1991), however there is considerable uncertainty as to how selective the method is for apoptotic cells. The method is certainly not specific for apoptotic cells, since any DNA strand breaks should in theory label, whether they are present in necrotic cells or in cells in the course of mitosis. Observations clearly show that cells with non-apoptotic morphology label with this method. Caution is therefore necessary in interpretation, and examination of the morphology of the labelled cells is essential (Ansari et al., 1993; Gold et al., 1993; Wijsman et al., 1993).

The method needs to be carefully optimized for individual situations, especially with regard to fixation, permeabilization and protease digestion, but can be used in conjunction with automated image cytometry (Wijsman et al., 1993) or flow cytometry (Gold et al., 1993). While some investigators have reported that proliferating cells are not labelled by *in situ* nick translation methods (Gold et al., 1993), others show that single strand DNA breaks can be labelled in dividing cells at the single cell level (Patkin et al., 1994). To investigate the relationship between apoptosis and proliferation in a cell population, it is possible to combine ISNT with bromodeoxyuridine (BrdU) incorporation in a double labelling technique (Mundle et al., 1994).

b. TUNEL

The TUNEL technique (a re-discovery of a method first described by Modak and Bollum in 1970 and 1972), identifies nuclei in locations where programmed cell death would be expected (Fig. 2), but labels cells with both apoptotic and non-apoptotic morphology (Gavrieli et al., 1992; Gorczyca et al., 1993). The interpretation of this observation is debatable, but opens the possibility that the technique labels "pre-apoptotic" nuclei (Negoescu et al., 1996). The method is available with a number of modifications and enhancements from the original description, including the use of various fixatives, permeabilizations, nuclease digestion, and also in combination with immunocytochemical and *in situ* hybridization procedures (Wride et al., 1994; Li et al., 1996; Negoescu et al., 1996; Sträter et al., 1996; Umemura et al., 1996; Sanders et al., 1997). Increased resolution has been claimed using bromodeoxyuridine-labelled UTP and oligonucleotides (Li and Darzynkiewicz, 1995; Aschoff et al., 1996), and the method can be adapted for use with flow cytometry (Bromidge et al., 1995; Yamada and Hata, 1995).

The method is not without its potential problems, however, and morphological confirmation of apoptosis remains necessary (Cervós-Navarro and Schubert, 1996).

Both the TUNEL and the ISNT methods label both apoptotic and necrotic cells, but possibly with different temporo-spacial nuclear staining patterns (Kressel and Groscurth, 1994). Although some investigators report that TUNEL does not discriminate between apoptosis and necrosis (Grasl-Kraupp et al., 1995), others contend that the early stages of apoptosis are preferentially labelled by TUNEL, while the early stages of necrosis are preferentially labelled by ISNT, and that TUNEL is generally more sensitive for apoptosis than is ISNT (Gold et al., 1994). This has been controversial, however, and the view has been expressed that both methods are equally sensitive and selective for apoptosis, while necrotic cells are also equally well labelled by both methods but at a lower sensitivity (Mundle and Raza, 1995). Direct comparison of the two methods appears to show that TUNEL indeed is generally more sensitive at detecting strand breaks than ISNT, and labels apoptotic cells at a higher intensity than necrotic cells (Gorczyca et al., 1993). Whether this reflects a greater number of strand breaks in apoptotic cells (Gorczyca et al., 1993) is unclear, since this would presumably depend on the stage of cell death examined because DNA degradation in necrosis, depending on lysosomal activity, is a relatively late event (Mundle and Raza, 1995). The consensus would appear to be that both methods need to be applied, and labelled cells need to be examined for morphological characteristics of apoptosis, for a rigourous demonstration of apoptotic, as opposed to necrotic, cells.

A very recent development has been the use of *in situ* hybridization, using poly(A) oligonucleotide probes, as an alternative to the TUNEL method (Hilton et al., 1997), but with similar sensitivity.

Some of the more controversial aspects of these techniques may be addressable using electron microscopical adaptations (Fig. 3). Both ISNT (Thiry, 1991; Migheli et al., 1995) and TUNEL (Nishikawa and Sasaki, 1995; Sanders and Wride, 1996) have been so adapted, and confirm that cells with

non-apoptotic morphology are labelled by these techniques.

5. DNA laddering

The "laddering" of ethidium bromide-stained DNA fragments on a gel is one of the characteristics of apoptosis (Compton, 1992; Bortner et al., 1995; Earnshaw, 1995). Bearing in mind that not all examples of apoptosis may be accompanied by early DNA breakdown (Schulze-Osthoff et al., 1994), the analysis of DNA degradation by examining the ladder has become a standard technique (Walker et al., 1993; Sgonc and Wick, 1994; Fournel et al., 1995). The method may be adapted for increased sensitivity by means of Southern blotting using a DNA probe (Wong et al., 1994).

This is, however, a technique that requires a large number of cells, and does not lend itself easily to situations in which apoptotic cells are scattered through a larger population of non-apoptotic cells. Also, it is not an *in situ* technique, so it cannot reveal the distributions of the apoptotic cells. In developmental situations, for example, where scattered apoptotic cells are the norm, the demonstration of a DNA ladder may be difficult (Zakeri et al., 1993), although end labeling may be helpful (Toné et al., 1994).

6. Flow cytometry

Flow cytometric analysis provides a means of quantifying the proportion of cells in a population that are undergoing apoptosis using intercalating or externally binding DNA dyes (see above), and in heterogeneous tissues this may be coupled with phenotypic markers expressed at the cell surface (Nicoletti et al., 1991; Swat et al., 1991; Hardin et al., 1992; Sgonc et al., 1994; Telford et al., 1994). A wide variety of both permeant (e.g. the Hoechst dyes, DAPI) and impermeant (e.g. ethidium bromide and propidium iodide) DNA dyes have been evaluated in the detection of apoptosis by this technique (Telford et al., 1992). More recently, new dyes have been developed which label apoptotic cells, and may be used either with flow cytometry or fluorescence microscopy, but which do not label living cells thus leaving them fully viable (Idziorek et al., 1995).

There has been discussion of the potential of this technique to discriminate between apoptosis and necrosis, taking advantage of the changes and differences in membrane permeability and chromatin condensation (Dive et al., 1992). Using the impermeant dyes ethidium bromide and propidium iodide, normal cells exhibit little or no staining, apoptotic cells exhibit low level staining, and necrotic cells exhibit bright staining (Telford et al., 1994). However, these distinctions are not always very clear, and attempts have been made to improve the discrimination using combinations of dyes (Telford et al., 1994).

The TUNEL method has also been used in conjunction with flow cytometry in place of the DNA

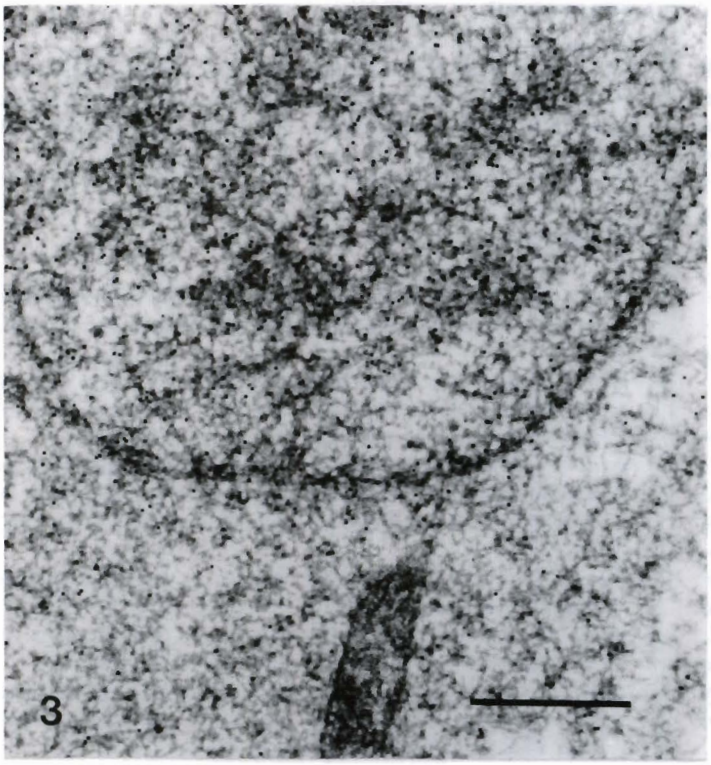

Fig. 3. Electron micrograph of a cell from the limb bud of a four-day chick embryo, showing the TUNEL technique at the ultrastructural level. The nucleus is heavily labelled with gold particles, particularly in the dense heterochromatin, while the cytoplasm shows only background labelling. See Sanders and Wride, (1996). Bar: 0.5 μm.

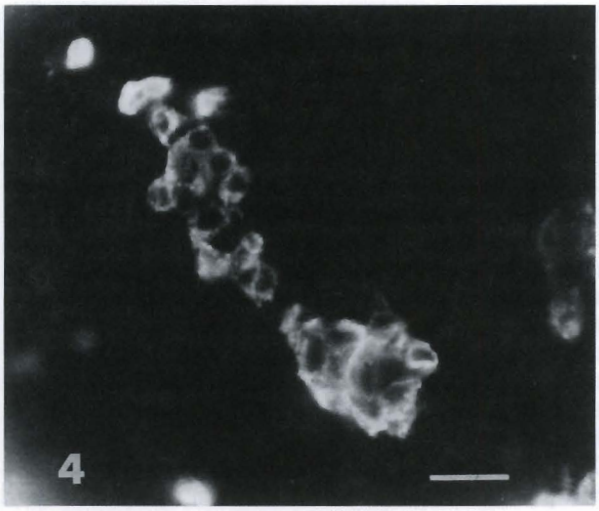

Fig. 4. Whole mount preparation of the epithelial sheet of cells from the limb bud of a four-day chick embryo, labelled with annexin coupled to fluorescein. Groups of cells in the epithelial sheet are fluorescent at their cell membranes, while other cells are not. The annexin has labelled phosphatidylserine which has been externalized on apoptotic cells. Bar: 100 μm.

binding dyes, and may offer a means of quantitatively estimating the degree of DNA strand breakage (Gorczyca et al., 1993; Bromidge et al., 1995; Yamada and Hata, 1995).

7. Gene expression

The altered expression of genes specifically associated with the process of apoptosis provides opportunities to identify cells in various stages of cell death. For example, there are monoclonal antibodies that appear to recognise cell surface determinants that are characteristic of cells undergoing apoptosis or engulfment (Rotello et al., 1994). Another example is the expression of the carbohydrate Le^y-antigen as a possible predictor of, or accompaniment to, apoptosis but not necrosis (Hiraishi et al., 1993; Minamide et al., 1995). Similarly, the relationship between the expression of *c-fos* and *c-jun* and the onset of programmed cell death may offer a corroborative method for identifying such cells (Smeyne et al., 1993; Estus et al., 1994).

The induction and activation of tissue transglutaminase appears to be closely linked to the onset of apoptosis in several tissues (Fesus et al., 1987; Piacentini, 1995). The function of transglutaminase is to form stable protein cross-linkings, but its mechanism of action in apoptosis is not clear. However, its immunocytochemical detection correlates with the appearance of apoptotic morphology in both developmental and pathological situations (Jiang and Kochhar, 1992; Piacentini et al., 1992; Pesce et al., 1993). The combination of transglutaminase immunocytochemistry with TUNEL provides good corroborative evidence for apoptosis (Polakowska et al., 1994).

8. Membrane asymmetry

The demonstration that apoptosis is accompanied early by changes in plasma membrane phospholipid symmetry affords a further means of examining this form of cell death. The shift of phosphatidylserine from the cytoplasmic face of the membrane to the external face results in a loss of membrane asymmetry which is believed to play a role in the recognition and removal of apoptotic cells by macrophages. This may be exploited in the study of apoptosis by means of the phospholipid-binding protein, annexin V (Fig. 4), which has high affinity for phosphatidylserine (Koopman et al., 1994; Martin et al., 1995; Vermes et al., 1995). The combination of fluorescently labelled annexin V, which binds to the cell surface, with propidium iodide or ethidium bromide, indicates that phosphatidylserine exposure at the surface coincides with chromatin condensation and precedes loss of membrane integrity.

9. Concluding remarks

Although apoptosis is characterized by many well defined cellular changes, such as: altered morphology, shrinkage, chromatin condensation, DNA fragmentation, new gene expression, and membrane asymmetry; it is clear that no single technique is adequate to unequivocally demonstrate apoptosis. The wide choice of methods now available provides ample opportunity to select two or three complementary techniques that are suited to any individual situation, and which together provide well substantiated evidence for the presence of apoptotic cells.

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