

Identification of Programmed Cell Death In Situ via Specific Labeling of Nuclear DNA Fragmentation

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Abstract. Programmed cell death (PCD) plays a key role in developmental biology and in maintenance of the steady state in continuously renewing tissues. Currently, its existence is inferred mainly from gel electrophoresis of a pooled DNA extract as PCD was shown to be associated with DNA fragmentation. Based on this observation, we describe here the development of a method for the in situ visualization of PCD at the single-cell level, while preserving tissue architecture. Conventional histological sections, pretreated with protease, were nick end labeled with biotinylated poly dU, introduced by terminal deoxy-

transferase, and then stained using avidin-conjugated peroxidase. The reaction is specific, only nuclei located at positions where PCD is expected are stained. The initial screening includes: small and large intestine, epidermis, lymphoid tissues, ovary, and other organs. A detailed analysis revealed that the process is initiated at the nuclear periphery, it is relatively short (1–3 h from initiation to cell elimination) and that PCD appears in tissues in clusters. The extent of tissue-PCD revealed by this method is considerably greater than apoptosis detected by nuclear morphology, and thus opens the way for a variety of studies.

PROGRAMMED cell death (PCD)¹ is a selective process of physiological cell deletion (Wyllie, 1981; Umansky, 1982; Bursch et al., 1990; Ucker, 1991). Its execution plays a major role in the control of shape and size in normal and abnormal processes (Kerr et al., 1972, 1987; Martz and Howell, 1989; Williams, 1991; Ucker, 1991). PCD exerts a homeostatic function in relation to tissue dynamics, as the steady state of continuously renewing tissues is achieved by a balance between cell replication and cell death. While cells active in DNA synthesis can be specifically labeled in situ by [³H]thymidine, ¹²⁵IUdR, or BrdU incorporation, the lack of an appropriate methodology for the identification of PCD in situ hampers the research of tissue kinetics (Hume, 1987; Allen, 1987; Hirshfield, 1988; Rothenberg, 1990; Tadakuma et al., 1990; Motyka and Reynolds, 1991). Most of our present knowledge about the nature of programmed cell death has come from studies with cells in culture, mainly lymphocytes, in systems where PCD was experimentally induced (reviewed by Allen, 1987; Martz and Howell, 1989; Williams, 1991; McConkey et al., 1990; Ucker 1991). These experiments showed that PCD is associated with endogenous endonuclease activity (Wyllie, 1980; Wyllie et al., 1984; Cohen and Duke, 1983, 1984; McConkey et al., 1989, 1990; Shi et al., 1990; Brune et al., 1991). It seems that chromatin cleavage is the most characteristic biochemical feature of the process (Wyllie et al., 1984; Kerr et al., 1987; Martz and

Howell, 1989; Shi et al., 1990; Tian et al., 1991). Current detection methods involve genomic DNA extraction and analysis, which does not allow distinction between individual cells. Thus, the appearance of the ladder of nucleosomal DNA fragments in agarose gels became the hallmark of PCD.

Morphologically, PCD is known as apoptosis (Kerr et al., 1972). Its gross features, identified by EM, include nuclear chromatin condensation, compactness of cytoplasmic organelles, and the appearance of pedunculated protuberances on the cell surface (Wyllie et al., 1984; Kerr et al., 1987). In general, it is difficult to distinguish in situ cells undergoing apoptosis, by light microscopy (Kerr et al., 1987). Apoptotic cell appearance is limited to only a few minutes (Russell et al., 1972; Sanderson, 1976; Matter, 1979; Kerr et al., 1987), and apoptotic bodies in diverse forms are seen for only a few hours before they are phagocytized (Wyllie et al., 1980; Bursch et al., 1990). Moreover, DNA fragmentation can be found not only in histologically defined apoptotic cells, but also in morphologically intact cells going through the process of PCD (Umansky, 1982; Motyka and Reynolds, 1991), as also shown below.

In this work we describe the development of a method for an in situ labeling of DNA breaks in nuclei, in tissue sections processed through the routine procedure of histopathology, and its utilization for the study of tissue dynamics.

The method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA, ensuing a synthesis of a polydeoxynucleotide polymer. After the exposure of nuclear DNA on histological sections by pro-

1. *Abbreviations used in this paper:* PCD, programmed cell death; RT, room temperature; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

teolytic treatment, TdT was used to incorporate biotinylated deoxyuridine at sites of DNA breaks. The signal was amplified by avidin-peroxidase, enabling conventional histochemical identification by light microscopy.

The method of TdT-mediated dUTP-biotin nick end labeling (TUNEL) was tested on a variety of tissues in which the migration of cells to their final destination is already delineated unequivocally or in tissues that are known for their active PCD. The results demonstrate that in many of these examples, where traditional criteria of apoptosis are missing, the topographical arrangement of nuclei labeled for DNA breaks by TUNEL is in perfect agreement with the expected location of PCD. In addition to being a useful tool for the *in situ* identification of PCD, this method enables a quantitation of the process in cell populations.

Materials and Methods

Tissue Preparation

Tissue samples were fixed in 4% buffered formaldehyde, and embedded in paraffin. 4–6 μm paraffin sections were adhered to slides pretreated with 0.01% aqueous solution of poly-L-lysine (300,000 mol. wt.; Sigma Chemical Co., St. Louis, MO). Deparaffinization was done by heating the sections for 10 min at 70°C, or 30 min at 60°C. Hydration was done by transferring the slides through the following solutions: twice to xylene bath for 5 min, and then for 3 min to 96% ethanol twice, 90% ethanol, 80% ethanol, and double distilled water (DDW). Fresh solvents are recommended as traces might interfere with the enzymatic reaction.

Thymocyte Preparation and Dexamethasone Treatment

Male Sabra rats, 3–4-wk old (~ 50 g), were sacrificed by decapitation. The thymus gland was removed, washed in PBS, and then placed in 4°C cold RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The thymus tissue was minced and the cells were separated by a metal pins comb. The cell suspension was filtered through 250- μm metal mesh and centrifuged for 4 min at 400 g, at room temperature (RT). The supernatant was discarded, and the pellet was resuspended in 37°C RPMI 1640. Cell concentration was adjusted to 4×10^7 cells/ml. Dexamethasone was added to the cell culture to a final concentration of 1 μM (from a 2 mM stock solution in ethanol, Sigma). Cells were cultured in a humidified incubator under an atmosphere of 5% CO₂ in air, 37°C, for 1.5, 3, 4.5, and 6 h. The control culture was incubated without dexamethasone for 6 h. 2–3 ml of the cell culture were processed for PCD staining and from the remaining of the culture (10–20 ml) the DNA was extracted.

Thymocyte Preparation for Cytology

At the end of the incubation cells were centrifuged for 4 min, 400 g, at RT. Most of the supernatant was discarded, and the pellet was resuspended in the remaining medium. 4% buffered formaldehyde was added to the suspension brought to the original volume, and kept at RT. A drop of the cell suspension was put on slides precoated with 0.01% poly-L-lysine and air dried.

DNA Nick End Labeling of Tissue Sections

Nuclei of tissue sections were stripped from proteins by incubation with 20 $\mu\text{g/ml}$ proteinase K (PK) (Sigma Chemical Co.) for 15 min at RT, and the slides were then washed in DDW for 2 min, four times. Endogenous peroxidase was inactivated by covering the sections with 2% H₂O₂ for 5 min at RT. The sections were rinsed with DDW, and immersed in TDT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TDT (0.3 e.u./ μl) and biotinylated dUTP in TDT buffer were then added to cover the sections, and then incubated in humid atmosphere at 37°C for 60 min. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at RT. The sections were rinsed with DDW, covered with 2% aqueous solution of human serum albumin (HSA), or BSA for 10 min at RT, rinsed in

DDW, and immersed in PBS for 5 min. The sections were covered with Extra-avidin Peroxidase (BioMakor, Rehovot, Israel) diluted 1:10–1:20 in water, incubated for 30 min, 37°C, washed in DDW, immersed for 5 min in PBS, and stained with AEC for about 30 min at 37°C.

DNA Nick End Labeling of Thymocytes (Cell Suspension)

Slides with cell culture preparations were treated as tissue preparations, except the following: (a) No deparaffinization was needed; (b) the incubation with PK was omitted.

DNAase Treatment In Situ

Sections were deparaffinated, processed through PK, and peroxidase inactivation as described. The sections were then pretreated with DN buffer (30 mM Trizma base, pH 7.2, 140 mM K cacodylate, 4 mM MgCl₂, 0.1 mM DTT). DNAase I (1 $\mu\text{g/ml}$ –100 ng/ml; Sigma Chemical Co.) dissolved in DN buffer was added to cover the section. After a 10-min incubation at RT, the slides were washed extensively with DDW, and continued to be processed through DNA nick end labeling.

DNA Extraction

At the end of each incubation period, the thymocyte culture was centrifuged for 4 min, 400 g. The pellet was resuspended in a lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA, 100 $\mu\text{g/ml}$ PK, 1% SDS), and incubated at 37°C, till the mixture became clear. 100 $\mu\text{g/ml}$ PK was added every few hours. The DNA was extracted by phenol/chloroform 1:1 (Chloroform-chloroform/isoamyl alcohol 24:1), method, precipitated overnight in –20°C ethanol containing 0.3 M final concentration NaAcetate, and centrifuged for 30 min, 4°C, at 12,000 g. The pellet was resuspended in TE buffer (0.1 M Tris-HCl, pH 8.0, 10 mM EDTA). The DNA was subsequently treated with RNAase (100 $\mu\text{g/ml}$) for 1 h at RT, incubated overnight with PK (100 $\mu\text{g/ml}$) at 37°C, and finally reextracted with phenol, phenol/chloroform, chloroform, precipitated with ethanol, and resuspended as described above.

Agarose Gel Electrophoresis

DNA samples, of ~ 0.2 μg each, were electrophoretically separated on 1.8% agarose gel containing ethidium bromide (0.4 $\mu\text{g/ml}$). DNA was visualized by a UV (302 nm) transilluminator and the gels were photographed with a polaroid camera.

Results

The feasibility of PCD labeling *in situ* by the TUNEL method was first tested in the small intestine. The epithelium of this tissue has a high turnover rate. It is a simple columnar epithelium with a typical kinetics related to its unique architecture. Cells proliferate at the lower part of the crypt and migrate up the villus towards the lumen. As the cells move, they differentiate, senesce, and finally shed to the lumen (summarized by Wright and Alison, 1984).

TUNEL clearly revealed a distinct pattern of nuclear staining (Fig. 1, A and C). There was a marked labeling at the tips of the villi, which faded at cells located more distally to the gut's lumen. There seems to be also an internal gradient with respect to the reaction intensity. Where the staining was light, it was confined to the nuclear periphery. As the labeling intensified it expanded also to the nucleus center (Fig. 1 C).

The staining is typically confined to a cluster of neighboring villi while other areas of the mucosa remain unstained. This pattern of mucosal patches was reproducibly demonstrated in preparations from human, rat, and mouse (Fig. 2).

The proteolytic pretreatment enhances the TdT reaction considerably and was usually essential while the omission of

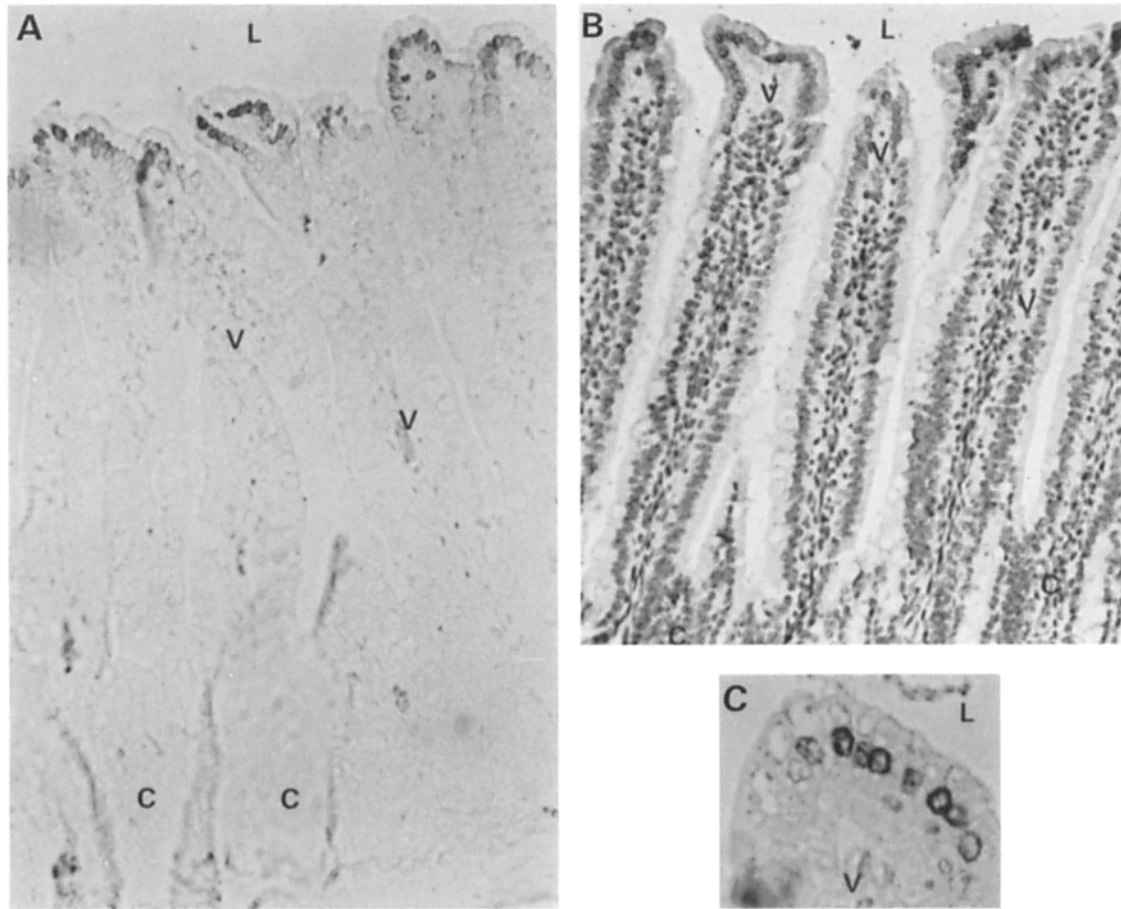


Figure 1. TUNEL of rodent small intestine. Paraffin sections of rat ileum, TUNEL stained: (A) Endogenous DNA fragmentation in nuclei indicating PCD. (B) After pretreatment with DNAase I (100 ng/ml). (C) A higher magnification of villus tip, TUNEL stained. Gut lumen (L), villi (V), and crypts (C) are indicated.

either TdT or its biotinylated substrate gave completely negative results (Fig. 3).

Never, in any of the many preparations that were carefully screened, were nuclei at the crypt labeled.

Nuclear Staining following DNAase Treatment

Histological sections of the small intestine, pretreated with PK, were exposed to DNAase I, and the nicks were then end-labeled with TdT-dUTP (Fig. 1 B). The control was a con-

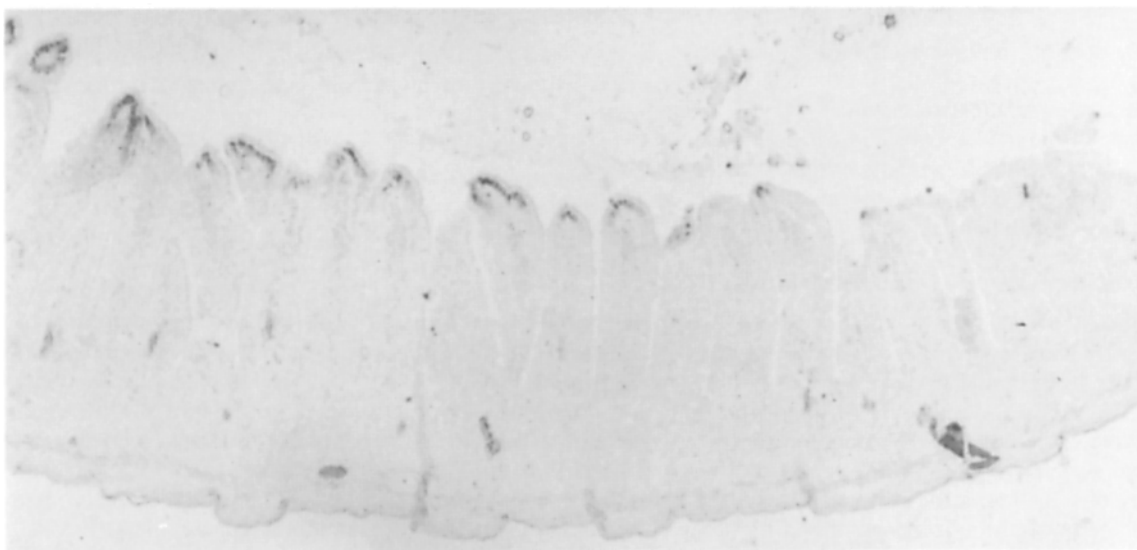


Figure 2. Regional TUNEL staining in the small intestine. Paraffin section of mouse ileum depicting the borderline between TUNEL reactive and non-reactive villi.

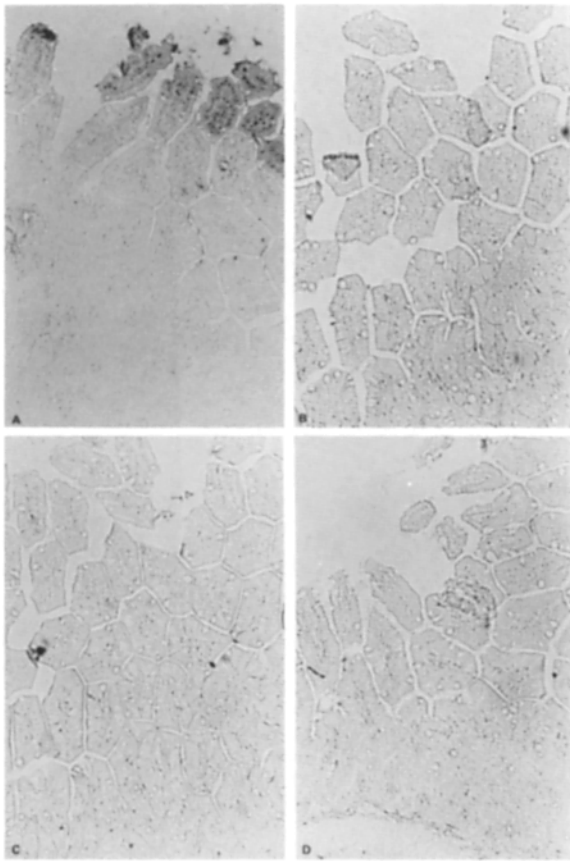


Figure 3. Controlled addition of reagents to the TUNEL staining. Serial paraffin sections of mouse ileum were TUNEL stained according to the complete procedure (A) or include the following modifications: minus TdT (B); minus biotinylated dUTP (C); minus ExtraAvidin-peroxidase (D).

secutive section which was DNA end-labeled without DNAase treatment (Fig. 1 A). TUNEL showed that pretreatment with DNAase I causes an intensive staining of all nuclei in the preparation. Quantitatively, there was a dose response relationship between DNAase concentration and the intensity of the labeling.

As seen from the staining following DNAase treatment, the accessibility to the enzymes is similar in all nuclei of all sections from a variety of different tissues.

The size and shape of the nuclei stained at the tips of the villi is similar to those stained in other parts of the tissue after DNAase treatment (Fig. 1 B). Hence, the staining of the PCD nuclei can not be attributed to pyknosis.

PCD Induction in Thymocytes by Dexamethasone

To follow the process stepwise and compare our new methodology with an established system, PCD was induced in thymocytes in culture, by dexamethasone (Cohen and Duke, 1984; McConkey et al., 1989a; Walker et al., 1991). In these studies, PCD was traced via overall DNA fragmentation as detected by agarose gel electrophoresis. We compared the results obtained by this method and TUNEL. Thymocyte cultures were incubated with dexamethasone for 1.5, 3, 4.5, and 6 h. The control were thymocytes incubated without dexamethasone for 6 h. At the end of the incubation period a cell sample was fixed and processed for TUNEL. From the

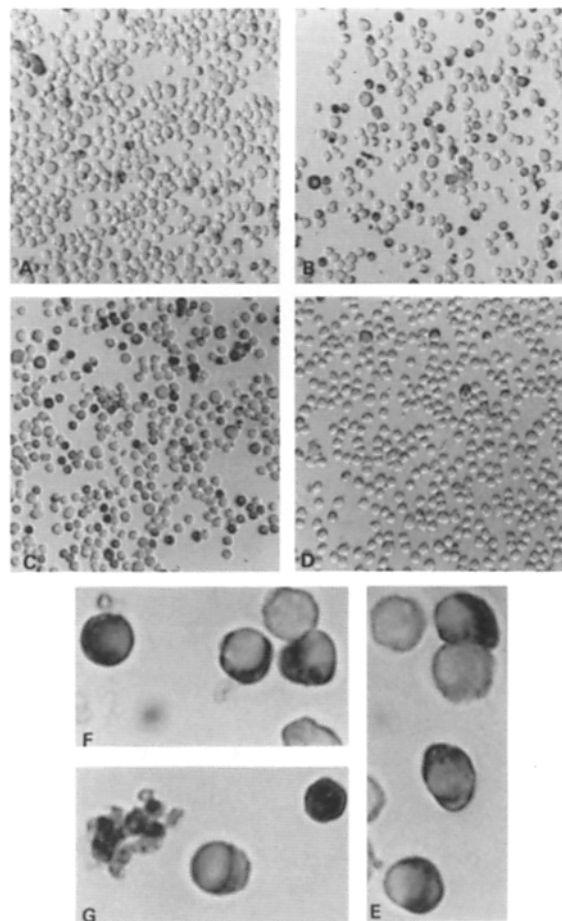


Figure 4. TUNEL staining of dexamethasone-induced PCD in thymocytes in vitro. Thymocytes were isolated from thymus glands of 3 week old male Sabra rats, and cultured at 4×10^7 cells/ml in RPMI 1640 in the presence of dexamethasone ($1 \mu\text{M}$) for : (A) 1.5 h, (B) 3 h, (C) 6 h, and (D) for 6 h without dexamethasone as control. Higher magnifications illustrate individual cells incubated for 1.5 h (E), 3 h (F), or 6 h (G) with dexamethasone. At the end of the incubation period 2–3 ml of the cell culture were fixed in 4% buffered formaldehyde and spread on polylysine coated slides. From the remaining, DNA was extracted.

rest of the culture DNA was extracted and run on agarose gel.

TUNEL enables the distinction of three features (Fig. 4): (a) A considerable fraction of the thymocytes are resistant to the dexamethasone treatment; (b) the intensity of staining varied with time; and (c) a unique pattern of staining is observed in individual nuclei. TUNEL of the control thymocyte cultures at time 0 (not shown) or after 6 h without dexamethasone (Figs. 4 D and 5 A) confirms that a substantial cell death (10–15%) already takes place in the thymus in vivo (Tadakuma et al., 1990). 1.5 h of incubation with dexamethasone increases the fraction of stained nuclei to 20%, and the rise in the percentage of stained nuclei continues until 3 h of incubation (Fig. 5 A). A longer incubation with dexamethasone did not increase the number of stained nuclei, but increased the intensity of staining in many of them (Fig. 4). In comparison, an agarose gel electrophoresis of DNA extracted from the thymocyte cultures (Fig. 5) shows that there is a DNA fragmentation only after 3 h of incubation with

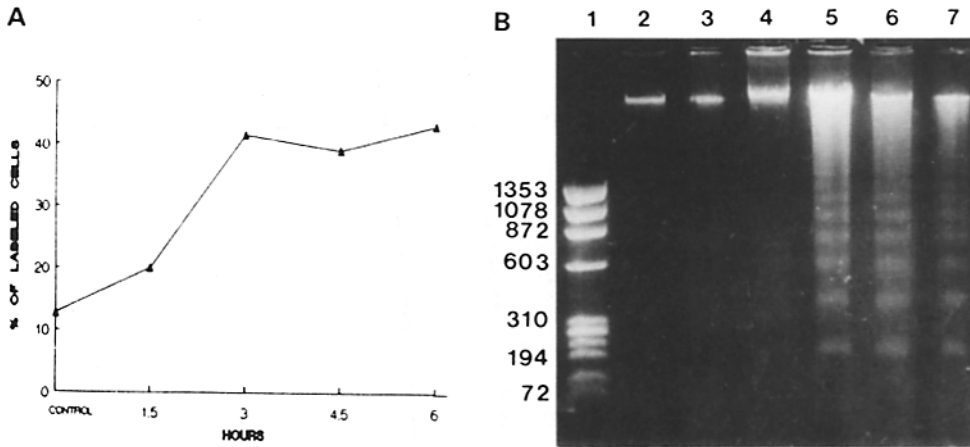


Figure 5. Kinetics of dexamethasone induced PCD in thymocytes: TUNEL versus gel electrophoresis. (A) Percentage of TUNEL stained nuclei of the thymocytes population in the preparations depicted in Fig. 2. Around 200 cells were counted, in randomly selected fields, for each point. (B) DNA analysis by 1% agarose gel electrophoresis of the genomic DNA extracted from the thymocytes culture incubated: (2) without dexamethasone for 6 h, or with dexamethasone for (3) 0 h, (4) 1.5 h, (5) 3 h, (6) 4.5 h, and (7) 6 h. (1) ϕ x DNA as a molecular marker.

dexamethasone according to the nucleosomal ladder (Fig. 5 B). Longer incubation with dexamethasone did not change the result. In another experiment, thymocytes were treated with either dexamethasone or sodium azide 0.1% (15 mM), an inhibitor of oxidative phosphorylation which causes cell death without DNA fragmentation (Nicoletti et al., 1991; Shi et al., 1990). No change in the percent of TUNEL positive cells was observed after a 6-h incubation with azide, in contrast to a 30% increase in PCD in cells exposed to dexamethasone (data not shown). In addition to the quantitative changes revealed by TUNEL, a change in the pattern of staining was also observed (Fig. 4). At 1.5 and 3 h of incubation, the staining in many of the nuclei has a crescent shape pattern. In longer incubations this crescent expands till the whole nucleus is stained, and eventually becomes condensed and fragmented (Fig. 4, E, F, and G).

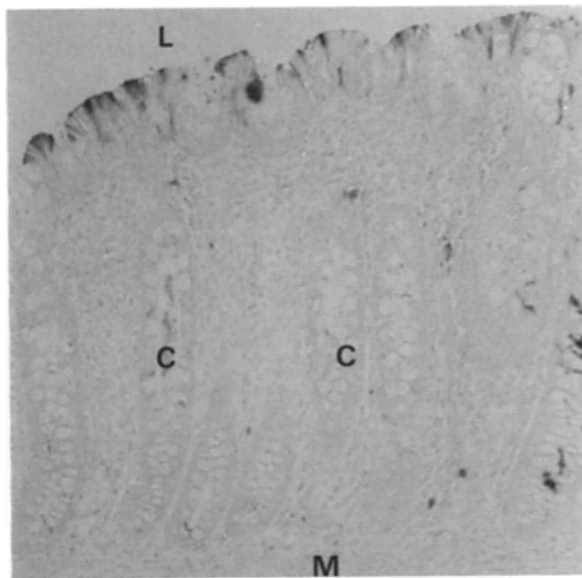


Figure 6. TUNEL of human large intestine. Paraffin section from a human large intestine stained by the TUNEL method for PCD. The gut lumen (L), the crypt (C), and the muscularis mucosa (M) are indicated.

PCD in Other Fast Renewing Tissues

The large intestine has a typical topographical arrangement, similar to the small intestine. Cells proliferate within the lower half of the crypt, migrate up, and finally shed to the gut's lumen. TUNEL of human or rodent large intestine stains only the uppermost nuclei at the edge of the crypt facing the lumen, while the rest of the crypt remains unstained (Fig. 6).

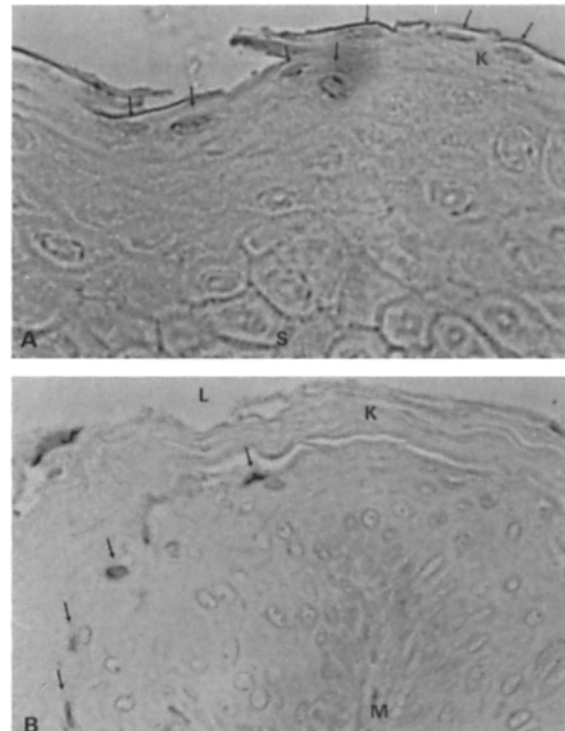


Figure 7. TUNEL of epidermal tissues. Paraffin sections of: (A) Human epidermis. (B) The rodent esophagus's keratinized stratified squamous epithelium. TUNEL stained PCD flattened nuclei are marked by arrows. The esophagus lumen (L), the keratin layer (K), the stratum spinosum (S), and the basement membrane (M) are indicated.

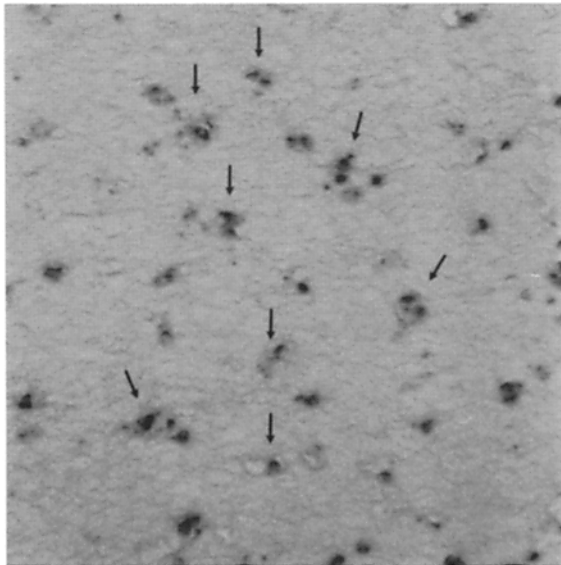


Figure 8. TUNEL of a lymphoid tissue. Paraffin section of TUNEL stained rat Peyer's patches. Aggregates of PCD cells are marked by arrows.

Epidermal Epithelium

The epidermis is also well characterized with respect to its tissue dynamics. The order of maturation in this stratified squamous epithelium is straightforward: the proliferative zone is at the basal layer and cells migrate toward the surface to become terminally differentiated. During this process, the cells acquire a flattened shape and their nuclei disintegrate as keratinization takes place. TUNEL stained only nuclei at the uppermost layer of this stratified epithelium, as evident in the human epidermis (Fig. 7 A). Similar results, obtained by DNA extraction and gel electrophoresis, were observed by McCall and Cohen (1991). The rodent esophagus is covered with keratinized stratified epithelium which is identical in its structure to the epidermis. TUNEL staining of the esophagus reproduces the results obtained with the epidermis (Fig. 7 B).

Extensive PCD in Lymphatic Tissues

The small intestine contains aggregation of lymphatic nodules called Peyer's patches. TUNEL stained the nuclei of cells in a dispersed pattern (Fig. 8). In many cases two to three adjacent nuclei are stained. Some of the nuclei are fragmented, exhibiting an advanced stage of apoptosis.

In the spleen, a large lymphatic organ, most of the cells in the region known as the white pulp are lymphocytes. Few nuclei at the white pulp were TUNEL stained in a scattered fashion. In contrast to the pattern in the Peyer's patches, the nuclei stained are isolated (not shown).

The thymus, where an extensive PCD is taking place, shows a unique distribution of TUNELed cells, a phenomenon which will be described elsewhere (manuscript in preparation).

PCD in the Ovary Clearly Marks Atretic Follicles

Ovarian follicles, each containing an oocyte, are embedded in the cortex of the ovary. Of the few hundred thousand folli-

cles present at birth, only a small fraction reach maturation. The rest are lost through a process known as atresia. TUNEL of ovary sections differentially stained the atretic follicles (Fig. 9). Nuclei of the granulosa cells surrounding the oocyte stained at different levels of intensity. For unknown reasons the cytoplasm of the granulosa cells and the oocyte itself have a nonspecific background staining, which does not exist in nonatretic follicles.

PCD in Slow Renewing Tissues

The probability of visualizing cells in PCD in tissues, where the turnover rate of the cell population is very slow, is very low. In few, such as the pancreas, the liver, and the kidney, PCD can occasionally be seen (Fig. 10). In the liver and kidney, in which the direction of tissue streaming is known, the location of TUNEL labeling is in agreement with the structural organization of the tissue (Arber et al., 1988).

Absence of DNA Fragmentation in Rapidly Sloughing Tissues

The endometrium consists of epithelium and lamina propria containing simple tubular glands that sometimes branch at their base near the myometrium. The endometrium can be functionally subdivided into two zones: (a) the functionalis which is renewed at each menstruation cycle; and (b) the basalis that is retained during menstruation and subsequently provides new epithelium and lamina propria for the renewal of the endometrium.

Cells with apoptotic morphology as defined by Kerr (1972), are easily identified in the endometrium, mostly in the lining epithelium. The nucleus of those cells is condensed, and the cytoplasm that surrounds it has a characteristic appearance. When human endometrium preparations from various stages of the menstrual cycle were PCD labeled, the nuclei of those apoptotic cells are stained distinctively. However, all other cells with normal morphology are not labeled. These apoptotic cells are never grouped, but rather dispersed randomly, and are generally scarce in the epithelium.

The Mammary Gland

Cessation of lactation causes rapid involution of the lactating epithelium within a few days. Although one would suspect this dramatic atrophy to be the result of massive PCD, no DNA fragmentation was detected by PCD labeling in histological preparation of the mammary gland of mice 1, 2, 3, 4, 5, and 6 d after weaning.

Discussion

At present, DNA fragmentation is considered the most characteristic feature of PCD as shown by studies of cells in culture (Martz and Howell, 1989; McConkey et al., 1990; Ucker, 1991). The drawback, however, is that the procedure used involved homogenization of the entire cell population and an analysis of the pooled DNA extract. The morphological definition of apoptosis is even less satisfactory since, according to these criteria (Kerr et al., 1987), PCD seems to be an occasional rare event. On the other hand, the TUNEL method, described above, is based on direct, specific, labeling of DNA breaks in nuclei, *in situ*.

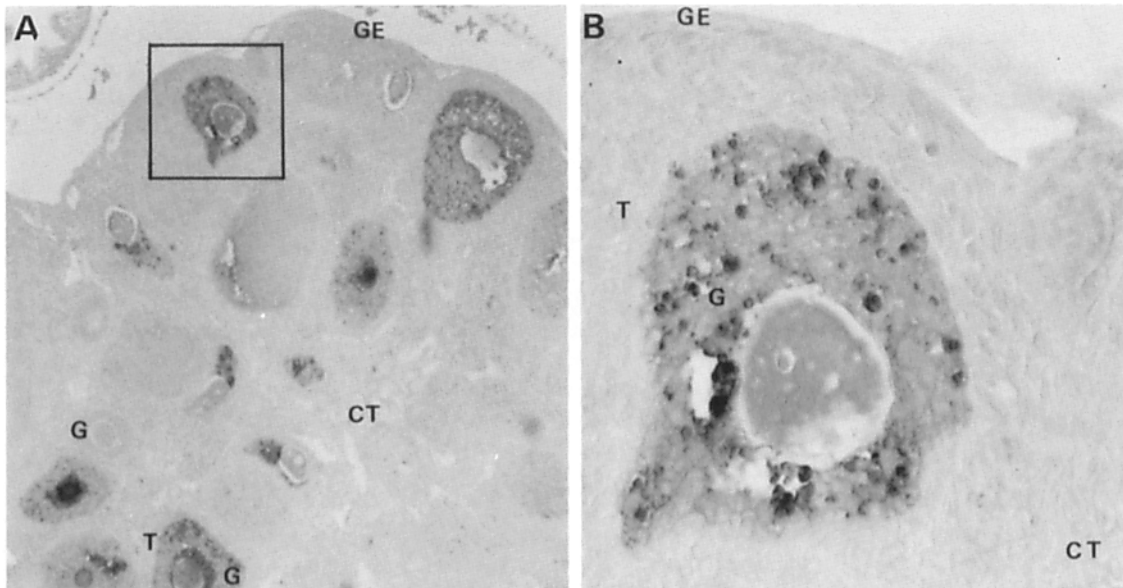


Figure 9. TUNEL of the ovary. Paraffin section of a mouse ovary. Atretic follicles are differentially stained. (A) Cross-section of the ovary. (B) Higher magnification of the atretic follicle marked by the rectangle in A, the oocyte was sectioned tangentially. Germinal epithelium (GE), connective tissue (CT), granulosa cells (G), and theca cells (T) are indicated.

Our prime objective in this work was to correlate the pattern of TUNEL staining in situ with data about systems in which PCD existence was already suspected through other means. The small intestine epithelium is typified by a high turnover rate (3–4 d for the replacement of the entire epithelium) and well-defined architecture of repetitive proliferating units where the location of cells which are about to be disposed, is known through pulse-chase experiments (summarized by Wright and Allison, 1984). This tissue was, therefore, selected first to test the validity of this method. Morphologically, no apoptotic cells were seen at the villi tips (Kerr et al., 1987). However, when stained for PCD by the TUNEL method, a clear demarcation of cell nuclei at the ex-

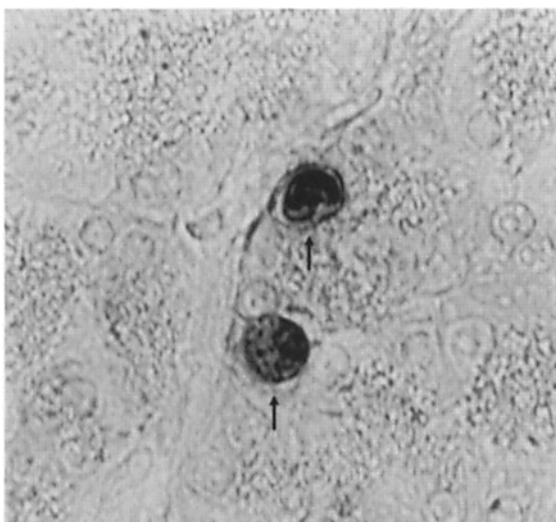


Figure 10. An example of TUNEL staining in slow renewing tissues. Paraffin section of a rat exocrine pancreas. PCD-positive cells are marked by arrows.

pected site is seen (Fig. 1 A). This staining cannot be attributed to a differential accessibility artefact, since potentially all nuclei in this tissue can react to TUNEL, as demonstrated by the results following DNAase treatment (Fig. 1 B). To our knowledge, this is the first report of DNA fragmentation as the last stage in the terminal differentiation pathway of epithelial cells in the G.I. tract. In other words, these cells are not “pushed to their death” by the underneath younger generation but rather prepare themselves for it by the activation of some internal signaling system.

A closer look at the pattern of TUNEL staining in the small intestine and other tissues provides further clues with respect to the fine details of the process: (A) DNA fragmentation is probably initiated at the nuclear periphery and progresses towards the center. This pattern was observed when PCD was followed in dexamethasone-treated thymocytes in vitro (Fig. 4, E and F). A nuclear-rim staining was also frequent among cells just entering PCD in the small intestine (i.e., in those more distal from the lumen, see Fig. 1 C); (B) PCD appears in clusters. In the small and large intestine a typical TUNEL staining is shared by a few tens of neighboring villi or crypts while cells facing the lumen outside the involved region do not react to the TdT (Fig. 2). An investigation of cross sections suggests that in the G.I. tract PCD occurs in waves of circular bands. Clustering of PCD was also observed in the epidermis; (C) the period through which dying cells can be revealed by the TUNEL method is relatively short. Our estimate ranges between 1 to 3 h and is based on the rate of accumulation and time of plateau of PCD in the thymocytes in vitro (Fig. 5). In addition, it can be inferred from the width of TUNEL-stained cell layers at the tips of small intestine villi, using the calculate rate of cell migration of 1.0–1.85 cell positions/h (summarized by Wright and Allison, 1984). Usually, the layer of TUNEL-stained cells at the villi tips is roughly equivalent to the cumulative amount of cells contained in the circumfer-

ence of one to four cell positions. This estimated 1–3-h time frame is in agreement with Kerr et al. (1987), who suggested a duration of only a few hours for the PCD process. In other words, the occurrence of PCD in slow renewing tissues should be a very rare event and even in tissues with a high turnover rate the identification of cells undergoing PCD could be compared with the probability of capturing cells in mitosis. Nevertheless, the transient nature of PCD should not obscure its importance.

The *in vitro* kinetics of thymocytes response to dexamethasone (Fig. 5) illustrates some of the quantitative aspects of the TUNEL method. While gel electrophoresis of DNA seems to provide a qualitative answer, whether the PCD process is taking place or not, TUNEL enables a determination of the actual fraction of cells undergoing PCD. Moreover, at time 0, when small fragments of DNA are not detected yet on the gel, ~10% of the cells are already stained for PCD by TUNEL and after 6 h the process reaches a plateau at ~40% (Fig. 5). These findings suggest that fully degraded nuclei are cleared from the thymus and that after a certain point in time the change in gel electrophoresis pattern reflects only the ongoing intracellular activity of the putative endonuclease(s). In addition, the extensive PCD observed in Peyer's patches (Fig. 8) confirms the results of Motyka and Reynolds (1991) who demonstrated a massive apoptosis in this lymphoid tissue which acquires an exceptionally high turnover rate.

In most of the tissues studied, the cells in which PCD was triggered are eliminated shortly thereafter: in the G.I. tract they are shed to the lumen, in the epidermis the cells as such disintegrate and become part of the keratin layer, and in the thymus the nuclei become fragmented and are canulated away from the thymus by a special system (manuscript in preparation). The only exception observed, so far, is the ovary in which apoptotic cells in atretic follicles might stay longer *in situ* with their degraded nuclei. Therefore, the ovary could serve as a reliable positive control for those interested in practicing the TUNEL method.

Interestingly, in tissues where a massive sloughing of the epithelium is taking place, such as the endometrium during menstruation or the mammary gland after weaning, we were not able to identify systematic PCD by our method except for staining in occasional apoptotic cells. Probably, in these tissues, the operating mechanism is primarily an overall ischemia.

Although the TUNEL method was independently developed, we learned later that this approach was applied in the past in a restricted number of tissues, using cumbersome autoradiography (Modak and Bollum, 1972; Appleby and Modak, 1977) and probably was, therefore, abandoned. The present technique introduces several apparent improvements with respect to reproductibility, resolution, and simplicity which enable it to be widely used in cytology, histology, and pathology. A similar combination of reagents was used to identify DNA breaks in differentiating myotubes (Dawson and Lough, 1988).

Nevertheless, with all the resolution power of this method, it should not obscure the full spectrum of phenotypic expressions of PCD. At present, DNA fragmentation is our best available criterion, but this does not imply that *in vivo* it proceeds all the way to the formation of a nucleosomal-size ladder of DNA. Moreover, in one of the best characterized ex-

amples of PCD, in *Caenorhabditis elegans*, it was shown that nuclease activation is a very late, independent, event in the process (Hedgecock et al., 1983). In fact our preliminary results indicate the absence of massive DNA fragmentation in some embryonal tissues where PCD allegedly takes place during the course of development.

In conclusion, the method described here offers several advantages *vis-à-vis* current approaches to PCD research. While compatible with the notion that in many cases PCD involved DNA fragmentation, the TUNEL method enables, in addition, *in situ* visualization of the process at the single-cell level. TUNEL staining precedes (and, therefore, does not depend on) the appearance of the nucleosomal ladder in gel electrophoresis (Fig. 5) and can potentially lead to the elucidation of ultrastructural aspects of the process. A study of PCD in cases where the steady-state of the tissue is impaired, either physiologically or pathologically, is underway.

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