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Progression of Apoptotic Cells by Pretreatment of Proteinase K

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Apoptosis can be difficult to detect in routine histological sections. Since extensive DNA fragmentation is an important characteristic of this process, visualization of DNA breaks could greatly facilitate the identification of apoptotic cells. Several techniques for the qualitative and quantitative detection of this process have been established; recently, an *in situ* nick end-labelling technique based on the detection of DNA fragmentation, which is a molecular characteristic of apoptotic cell death, was described. Applying this method to paraffin sections of rat tissues, sensitivity was observed to be inconsistently low with regard to the expected number of apoptotic cells. I describe a new modified method for formalin-fixed, paraffin-embedded tissue sections, protease pretreatment to permeate the tissue sections that involves an TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) is acknowledged as a method of choice in the rapid identification and quantification of the apoptotic cell fraction in paraffin tissue preparations. TUNEL was performed without apoptosis and with apopotosis samples to each of the three concentrations of proteinase K (10, 25, 40 mg/ml) pretreatments. In this study, I show that chemical pretretments of the tissue sections in proteinase K (25 mg/ml for 15 min at room temperature) considerably enhances the sensitivity of this nick end labelling technique.

Key Words: Apoptosis, Proteinase K, TUNEL, Rat, Paraffin section

INTRODUCTION

Apoptosis is a morphologically distinct form of programmed cell death that plays an important role in the physiological elimination of cells without causing inflammation[1,2]. Thus, it occurs in almost all tissues and organs, especially in those with a high turnover rate of constituting cells[3]. Furthermore, it has become obvious that neoplastic growth is not necessarily due to an increased proliferation rate but may also arise at a normal proliferation rate through cell accumulation as the consequence of abnormally prolonged survival of a neoplastic clone[4]. Thus, growth kinetic studies in normal and neoplastic tissues have to consider both proliferation and cell death. While several methods for the detection of proliferation *in situ* have been established, techniques for the identification of apoptotic cells are still at the development stage[5].

A molecular hallmark of apoptotic cell death is irreversible degradation of genomic DNA induced by activation of endonucleases. As a result, DNA fragments of about 180~200 bp accumulate in the nucleus of the dying cell (reviewed by frondsand Wyllie 1991). Recently, a 747-mediated nick end-labelling technique was described[6] and has been proven to be helpful in the *in situ* detection of DNA fragmentation and, thus, of apoptotic cells. However, when this method was applied together with a similar *in situ* end-labelling method[7]. Some cells with a clear cut apoptotic morphology were not stained or tissues in which programmed cell death is supposed to occur failed to contain labelled cells[6,8]. This suggests that the sensitivity of both methods is suboptimal. TUNEL, or terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling, is acknowledged as a method of choice in the rapid identification and quantification of the apoptotic cell fraction in cultured-cell preparations. Indeed, despite the now well-known apoptotic cell morphotype accurately described by Kerr and Wyllie[1].

Many papers dependent to chemical pretreatments of the biological samples have been devised to improve TUNEL sensitivity. These involve the incubation TUNEL was performed without apoptosis and with apoptosis samples to each of the three concentrations of proteinase K (10, 25, 40 mg/ml) pretreatments.

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MATERIALS AND METHODS

1. Quantification of Apoptosis in Rat skins

The 300 gm rats were a complete nonapoptosis sample and apoptosis samples by apoptotic responsed to glucocorticoid incubation during 3 hr with 13 mM dexamethasone.

2. Tissue Processing

Specimens of rat and human tissue were fixed in buffered 4% formaldehyde for at least 16 hr and embedded in paraffin according to routine procedure. Sections of 5 μm thickness were mounted on Starfrost pre-coated slides [Knittel Glaser; Braunschweig (Germany)].

3. Pretreatments

After deparaffinization and rehydration, tissue sections were digested to enable the enzymatic interporation of nucleotides. An optional pretreatment consisted of heating the sections either in 1 M NaSCN or in 2x SSC (0.3 M NaCl and 30 mM Na-citrate, pH 7) at 80℃ for 20 min, followed by thorough washings in distilled water. Subsequently, sections were digested with Proteinase K (Boehringer Mannheim) on variations in concentration [10 mg/ml, 25 mg/ml, 40 mg/ml in phosphate buffer saline (PBS)], for 15 min at room temperature (RT).

4. TUNEL

I used TUNEL original laboratory protocol (Boehringer Mannheim *In Situ* Cell Death Detection Kit, Peroxidase (POD) an *in situ* cell detection kit from a supplier of apoptosis detection products. Our laboratory protocol was adapted from Gavrieli *et al.*[6], Gold *et al.*[9], and Migheli *et al*[10]. Background was diminished by preincubating samples with 3% bovine serum albumin (BSA), 20% normal bovine serum in PBS, 30 min at RT. The specimens were then exposed, for 1 hr at 37℃ in a moist chamber, to the TUNEL labeling mix containing 0.135 U/μl calf thymus terminal deoxynucleotidyl transferase (TdT), 0.0044 nmol/μl digoxigenin-11-2'-deoxyuridine-5'-triphosphate (DIG-dUTP), and 1 mM cobalt chloride in 1X reaction buffer in distilled water. All reagents in the TUNEL mix were obtained from Boehringer Mannheim. After washing (4 PBS baths of 5 min each at RT), the specimens were resaturated (30 min at RT) in 3% BSA, and 20% normal sheep serum in 1% (w/v) Blocking Reagent (Boehringer Mannheim) in 0.1 M Tris-buffered saline (TBS). They were then treated for 1 hr at RT with a 1/120 (1.25 peroxidase U/ml) dilution of peroxidase-labeled anti-digoxigenin sheep Fab fragment (Boehringer Mannheim), followed by washing and a 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) color reaction.

RESULTS

1. Pretreatment

Staining results depended on the degree of digestion of the tissue sections. Protease digestion was necessary to make the DNA accessible for incorporation of nucleotides and was initially done with proteinase K. Protease treatment was considered inadequate when nuclei in the deeper parts of DNAse treated control sections remained unstained (Fig. 1c), whereas too strong digestion was characterized by poor morphology and faint staining of non-apoptotic nuclei (Fig. 1d). Proteinase K (25 mg/ml for 15 min) did better, with percentages reaching 50% for formalin fixed tissue.

No reaction product was detectable in non-apoptotic cells with dense chromatin, such as mitotic cells or lymphocytes (Fig. 1a).

2. Localization of Staining

Intense staining was observed in nuclei and nuclear fragments with the morphological characteristics of apoptosis:. Apoptotic bodies of various sizes showed distinct staining. Large bodies occasionally remained unstained in the center, a phenomenon also observed in DNAse-treated sections. The effect diminished after stronger protease digestion, with a concomitant loss of morphology, indicating that the condensed chromatin of large bodies is poorly accessible. Remarkably, the cytoplasm of apoptotic cells was also often stained, which suggests leakage of DNA fragments out of the nucleus. It is not clear whether this represents a true biological phenomenon or an artifact resulting from tissue processing (Figs. 1).

DISCUSSION

Apoptosis was called as by programmed cell death, represents a critical control mechanism in morphogenesis and in normal tell turnover of adult tissues. Morphologically,

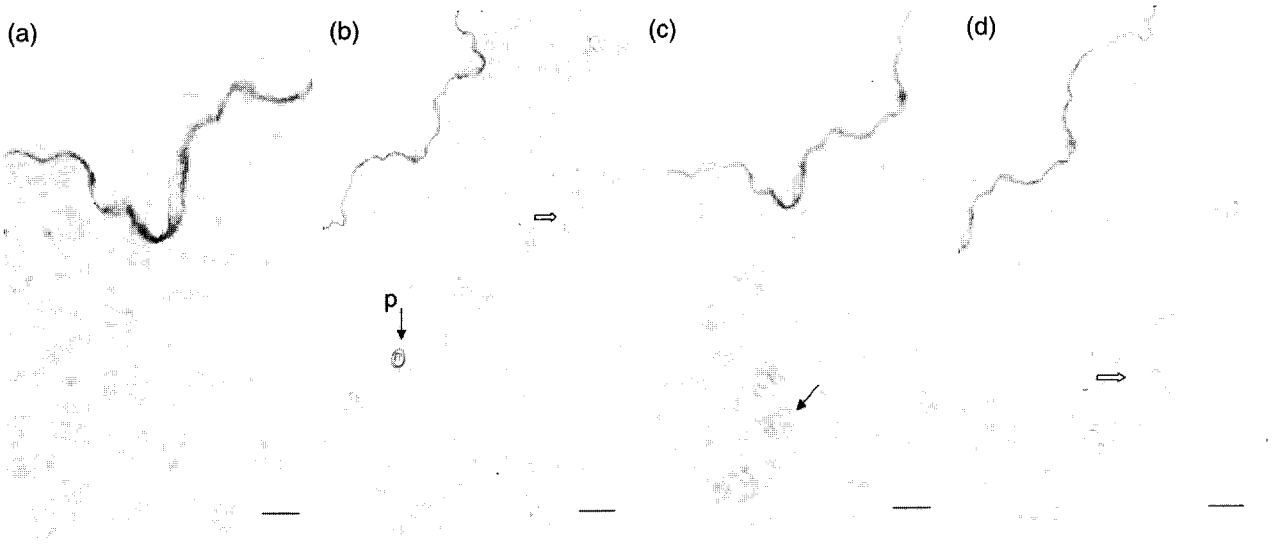

Fig. 1. The sections were digested with Proteinase K (Boehringer Mannheim) on variations in concentration (10 mg/ml, 25 mg/ml, 40 mg/ml in PBS), for 15 min at RT and negative control.
(a) Negative control of nonapotosis and **(b) (c) (d)** samples were induced to apotosis; **(b)** pretreatment Proteinase K 10 mg/ml **(c)** pretreatment Proteinase K 25 mg/ml **(d)** pretreatment Proteinase K 40 mg/ml. **(a)** was not showed apoptic feature, The presence of **(b)** in a nucleus initiating active cell death is shown by a mixed blue-brown staining (empty short arrow) and the TUNEL method stained condensed nuclei, micronuclei, and several normally shaped nuclei. The status of normally shaped, intensely TUNEL-positive cells corresponds to the stage at which high molecular weight DNA fragments can be detected, prior to changes in cell morphology. Because this staining was not delectable in negative controls, It must have resulted from specific incorporation of nucleotides. Proteinase K (25 mg/ml for 15 min) did better, with percentages reaching 50% for formalin-fixed tissue and paraffin section. Adjacent section was stained by TUNEL respertively. The TUNEL-stained sections, DAB-positive nuclei or bodies wire scored **(c)**. **(b)** and **(d)**, inadequate protease digestion: some cells remain. An typicall apoptotic body can still be discriminated (arrowhead). I observed both stained and unstained pyknotic nuclei and some-times staining of normal-looking nuclei. Cellular debris and nuclear ghosts(g)[11] showed faint.
Original magnification ×400. Bars = 10 μm

this process is characterized by cell shrinkage, loss of cell-cell contacts, and aggregation of the chromatin into dense. Cells rapidly fragment into membrane-bounded apoptotic bodies that contain intact organelles and condensed chromatin. They are phagocytosed by macrophages and neighboring cells or are shed into the lumen[1,5,11,14].

The aim of our study was to confirm the applicability of microwave irradiation, which is broadly used to enhancethe sensitivity of immunohistochemical procedures in paraffin sections. I wished to demonstrate improved staining results obtained by the TUNEL method[6] for the *in situ* detection of apoptotic cells.

Apoptosis is known to occur in considerable numbers of cortical thymocytes as a result of immunoselection[15] and is supposed to be involved in the physiological loss of enterocytes within the surface epithelium of the gut[6,7].

Thus, Rat skin were examined. Short-term heating of the tissue prior to proteinase K digestion clearly enhanced both the number of apoptotic cells detected and the staining intensity of the labelled nuclei in comparison with proteinase treatment alone.

In the present study, Scientists demonstrate that the ISEL procedure can label fragmented DNA in paraffin sections and that this method is useful to detect apoptosis. The technique combines the biochemical characteristic of DNA fragmentation with the typical morphology. The cytochemical signal greatly enhances recognition of scattered apoptotic cells and avoids confusion with other cells that have condensed chromatin, e.g., the telophase of mitotic cells or polymorphic leukocytes. Moreover, it enables automated image cytometry. In contrast to the biochemical method of measuring concentrations of low molecular weight DNA relative to total DNA[16] (to be distinguished from the qualitative analysis of DNA fragments by gel-electrophoresis), quantification of apoptosis by ISEL can be done on a cell basis with preservation of topological information. In tissue sections, several methods have been applied to facilitate the detection of apoptotic cells. e.g., enhancing the difference in staining intensity between condensed and normal chromatin by using semithin, resin-embedded sections[17].

DNA fragments enlarging during the apoptotic process. Prolonged microwave treatment (10 min or more) prior to tissue digestion led to a decrease in specific staining intensity, possibly due to pretreatment-induced release of small DNA fragments. In conclusion, short-term microwave irradiation of formalin-fixed tissue is suitable for the *in situ* detection of apoptotic cells, since the sensitivity of the TUNEL method is considerably enhanced.

To enhance uniform digestion of the tissue, I evaluated the effect of incubating the sections in the chaotropic agent NaSCN or in 2×SSC at 80℃ before protease treatment. This has been shown to improve the rerults of *in situ* hybridization studies on paraffin sections, as it may loosen the bonds between nucleic acids and proteins[18].

There are at least two reasons for preferring the labeling-assisted identification: The process is rapid: 2 to 5 minutes for chromatin condensation and cell fragmentation, 3 hours for apoptotic body phagocytosis[19,20]; The apoptotic cell has broadened and gained certain nuances: the apoptotic nucleus may condense without splitting (pyknotic nuclei;[2,21]) and, in the early stages of apoptosis, the nucleus may be slightly enlarged while DNA is starting to fragment[22]. Moreover, nuclear morphology is strongly dependent on sample fixation, with consequent effects on the identification and interpretation of the morphological aspects of apoptotic cells. The accessibility to DNA breaks for enzymatic reactions is reduced by the nuclear protein environment[23] and impaired by cell fixation currently performed with bridging aldehydes, paraformaldehyde[9], or formaldehyde[6], and sometimes followed by an ethanol postfixation[24]. Thus, chemical pretreatments of the biological samples have been devised to improve TUNEL sensitivity. These involve the incubation of cells with a detergent, most frequently Triton® X-100[22,25], which, in our experience, exhibited limited efficiency. Proteolytic enzymes, mainly proteinase K, have been used for tissue sections[9,10,23] but not yet applied to cultured cells. Since one of the effects of the widely used microwave antigen-retrieval method is peptide-bond cleavage[26], I assumed that this approach, together with proteinases, might prove efficient for TUNEL when applied to cultured cells. Two recent articles[27] reported a similar attempt on formaldehyde-fixed, paraffin-embedded, microwave-pretreated tissue sections.

TUNEL has been almost exclusively applied to cells fixed with paraformaldehyde or formaldehyde, the originally proposed fixatives. Pretreatments, of which microwaves proved the best, allowed TUNEL sensitivity to be extended over arange of fixatives without any notable impairment in specificity, permitting compromises between TUNEL and immunochemical labeling requirements. This was achieved with an original laboratory protocol. Effect of pretreatments on TUNEL. Tissues were fixed with formalin liquid, and TUNEL performed with the Boehringer Mannheim kit. TUNEL sensitivity is insufficient when performed without any pretreatment, regardless of the fixative used. Note that negative control tissue with apoptotic morphology are unlabeled. The best pretreatment condition, Proteinase K (25 mg/ml 15 min) did better, with percentages reaching 50% for formalin-fixed cells.

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