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Technical Advance

Biotin-Labeled Hairpin Oligonucleotides

Probes to Detect Double-Strand Breaks in DNA in Apoptotic Cells

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Hairpin oligonucleotides were synthesized with stems ending in a double-stranded structure, which can be ligated to double-strand breaks in DNA, and with loops that contain nucleotides modified by the attachment of biotin. These probes specifically and sensitively detect double-strand breaks in apoptotic cells. Localization of these probes is restricted to areas of chromatin characteristic of apoptosis, whereas much more diffuse labeling was obtained when all available 3′ DNA ends were labeled by terminal transferase. In principle, hairpin oligonucleotide probes can be designed with any type of 3′ or 5′ overhang complementary to double-strand DNA termini being detected. ***(Am J Pathol 1998, 152:897–902)***

Oligonucleotides modified by the incorporation of nonradioactive tags such as biotin or digoxigenin have been extensively used in chemical and biochemical procedures and in *in situ* labeling techniques.[1–3] In view of the current extensive interest in the process of apoptosis and its detection *in situ*, particularly in the analysis of pathological specimens, specific and sensitive probes are needed for the reliable detection of the molecular features of this form of cell death.[4–9] We showed that apoptotic cells in dexamethasone-treated thymus have double-strand breaks that can be ligated to double-stranded DNA fragments with 3′ overhangs or blunt ends.[10] Based on this observation, we designed oligonucleotides capable of detecting such double-strand breaks. These oligonucleotides are hairpins with stems ending in a double-stranded structure that can be ligated to double-strand breaks and loops that contain nucleotides modified by the attachment of biotin.

Materials and Methods

Preparation of Biotin-Labeled Hairpin Oligonucleotides

Oligonucleotides were designed with a 10-bp stem region to form a hairpin with a defined double-strand end (Figure 1). A loop of 20 nucleotides was designed to accommodate biotin labels without base pairing in this region. The oligonucleotide sequence is arbitrary and was designed to avoid misfolding of the hairpin into any structure other than that illustrated in Figure 1. At five places in the loop, the oligonucleotide was synthesized with amino modifier C6 deoxyuridine (Glen Research, Sterling, VA). After synthesis of the oligonucleotide, biotin was covalently attached to the amino groups by reaction with biotin bis-aminohexanoyl *N*-hydroxysuccinimide ester (Glen Research). The synthesis and postsynthesis biotinylation were performed by Synthetic Genetics Corp. (San Diego, CA). Alternatively, the hairpin was synthesized with the substitution of a preformed biotin-labeled thymidine analogue (Glen Research).

In Situ *Ligation of Labeled DNA Fragments on Tissue Sections*

Hairpin oligonucleotides were ligated to DNA in tissue sections *in situ* using T4 DNA ligase. Various tissues (described below) were used with the following protocol. Tissue fragments were fixed in either freshly prepared paraformaldehyde or buffered formaldehyde, with equivalent results, and were conventionally dehydrated and embedded in paraffin. The 6-μm sections were deparaffinized with xylene and rehydrated in graded alcohol

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concentrations. After washing in water, sections were incubated for 90 minutes at 65°C in 10 mmol/L sodium citrate, pH 6.0, and then rewashed with water.

The following procedures were performed at room temperature (23°C). Sections were incubated with 25 μg/ml proteinase K (Oncor, Gaithersburg, MD) in PBS for 5 minutes. This incubation time was used in the experiments reported here, but we found that it may need to be decreased or increased for optimization of signal and background in different tissues. A short treatment with proteinase K greatly increased the signal intensity. Long incubations with proteinase K increased background. Suppression of background binding depends on eliminating spurious ligase-independent binding of DNA, which is a negatively charged molecule, to the section. The preincubation in citrate buffer greatly reduced background. It is possible that other treatments may be necessary in tissues that have a greater density of positive charges.

Sections were then rinsed thoroughly with water. A mix of 50 mmol/L Tris/HCl, pH 7.8, 10 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 1 mmol/L ATP, 15% polyethylene glycol (8000 molecular weight; Sigma Chemical Co., St. Louis, MO), with hairpin oligonucleotide at 35 μg/ml and DNA T4 ligase (Boehringer Mannheim, Indianapolis, IN) at 250 U/ml, was added (20 μl per section). Sections were covered with glass coverslips and placed in a humidified box for 16 hours. The sections were then washed with several changes of water over 2 hours. Fluorescein-avidin conjugate (Vector Laboratories, Burlingame, CA) was added at 4 μg/ml in 50 mmol/L sodium bicarbonate, 15 mmol/L sodium chloride, pH 8.2, for 45 minutes. Sections were washed in the same buffer three times over 30 minutes and then in water for 20 minutes.

Terminal Deoxynucleotidyl Transferase

For the reaction of available DNA 3′ hydroxyls with terminal transferase, the published procedure was used[11] modified to accommodate the use of Texas Red as label rather than biotin. A mixture comprising 30 mmol/L Tris/HCl, pH 7.2, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride, 0.1 mmol/L dithiothreitol, 8 μmol/L Texas Red-X dUTP (Molecular Probes, Eugene, OR), and 800 U/ml terminal transferase (Boehringer Mannheim; 20 μl per section) was added for 1 hour at 37°C in a humidified incubator. After washing in water (two changes over 20 minutes), the sections were counterstained with the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) (1 μg/ml), mounted in Vectashield (Vector Laboratories), and observed by fluorescence microscopy.

Tissues

Thymus

Sprague-Dawley rats (150 g) were injected subcutaneously with 6 mg/kg dexamethasone (Sigma) dissolved in 30% dimethylsulfoxide in water.[12] Animals were killed after 24 hours.

Necrotic tissue

Sections from a Wilms' tumor from a 5-year-old male patient, containing extensive areas of necrosis, as often encountered in such tumors,[13] was used to provide samples with large numbers of necrotic cells.

Kidney

Mild trauma of the kidney was achieved by puncturing the capsule and loosening it away from the parenchyma. This resulted in a zone of apoptotic cells close to the site of trauma 24 hours later. Mild injury to the kidney results in apoptosis of epithelial cells associated with tubular degeneration.[14–16]

Results

Design of Oligonucleotides

Hairpin oligonucleotides that can be used to detect double-strand breaks were designed with the features illustrated in Figure 1. We designed and used probes with three different types of ends in these experiments: blunt-ended, single 3′ A overhang, and double 3′ NN overhang.

Double Labeling of DNA Strand Breaks with Hairpin Oligonucleotides and Terminal Transferase

To test the sensitivity and specificity of these oligonucleotide hairpin probes in labeling double-strand breaks in nuclei of apoptotic cells, we applied probes to sections taken from tissues in which apoptotic cells were present but which might also include cells undergoing necrosis, which may have double-strand breaks.[17] The tissues used were thymus from rats treated with dexamethasone, mouse kidney in which apoptosis was provoked by puncturing the capsule, and Wilms' tumor containing extensive areas of necrosis. A double-staining procedure was used enabling the sequential ligation of a hairpin oligonucleotide probe followed by the labeling of all available 3′ DNA ends, in double- and single-strand breaks, by terminal transferase. Biotin on the oligonucleotide probe was visualized with a fluorescein-avidin conjugate, and 3′ ends were visualized by addition of Texas Red-dUTP. The following experiments used a hairpin probe with a single A 3′ overhang (Figure 1).

Both forms of labeling were dependent on the presence of enzyme (ligase or terminal transferase); no nuclear signal was observed when enzymes were omitted from the reaction mix added to the sections. Figure 2 illustrates this point in the traumatized mouse kidney, where sporadic cells undergoing apoptosis were observed close to the site of injury. Apoptotic cells were specifically labeled with equal intensity by both techniques.

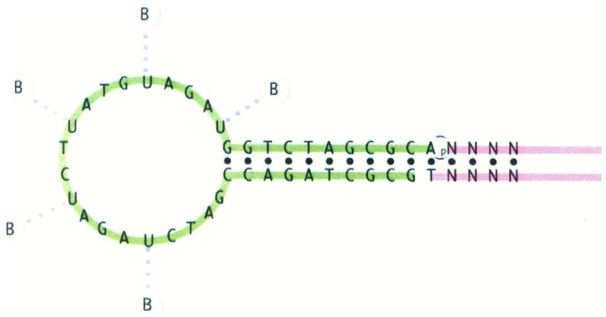

Figure 1. Biotin-labeled oligonucleotide probe for detection of double-strand breaks. Probes are hairpins designed so that the terminus of the stem has a characteristic structure, in this case a single 3′ A overhang. The loop contains five deoxyuridine derivatives labeled with biotin (B). The recessed 5′ phosphate of a double-strand break on the tissue section with a single T overhang can ligate to the 3′ A overhang on the probe. The 3′ overhang on the section does not ligate to the recessed 5′ hydroxyl on the probe because the oligonucleotide lacks a 5′ phosphate. This feature avoids the possibility of the probe ligating to 3′ hydroxyls on the ends of single-stranded DNA fragments.

The relative specificity of the labeling techniques for strand breaks in apoptotic and necrotic cell nuclei was tested by comparison of samples from dexamethasone-treated rat thymus, a tissue with large numbers of apoptotic cells,[12] and a sample of Wilms' tumor with extensive necrosis[13] (Figure 3). In the thymus, most cells undergoing apoptosis were labeled by both techniques, although a few appeared to be selectively labeled by terminal transferase or by ligation of hairpin probe. In the necrotic areas of the tumor, some cells were labeled by ligation of the hairpin probe. However, these cells were surrounded by much larger numbers of cells that were labeled to varying extents by terminal transferase. These cells appeared to be undergoing necrosis when these areas of the tumor were examined by conventional histology. The difference between the thymus and Wilms' tumor specimens was most evident when both fluorochromes were observed simultaneously with a dual-wavelength filter (Figure 3, c and f). Whereas most cells in the thymus appeared yellow, indicating equal labeling by both techniques, few cells in the tumor appeared yellow, indicating much greater labeling by terminal transferase than by ligation of hairpin probes.

Interestingly, observation of double-stained apoptotic cells by confocal microscopy revealed intranuclear patterns of labeling by the hairpin probe (Figure 4). Some cells had chromatin condensations or marginations that were equally labeled by hairpin probes and by terminal transferase; Figure 4, a–c, shows an example in the kidney. However, many cells in the thymus showed a zone of more intense ligase-dependent labeling around the periphery of the nucleus, whereas terminal transferase labeled these nuclei more uniformly. Although these differences might reflect limitations of the different techniques, it is also possible that they represent the concentrations of double-strand and single-strand breaks, respectively.

Different Types of Probes

Three types of probes (blunt, single-nucleotide, and double-nucleotide overhangs) were compared for their specificity and sensitivity. The patterns of labeling for the three kinds of probes were generally similar. However, the NN

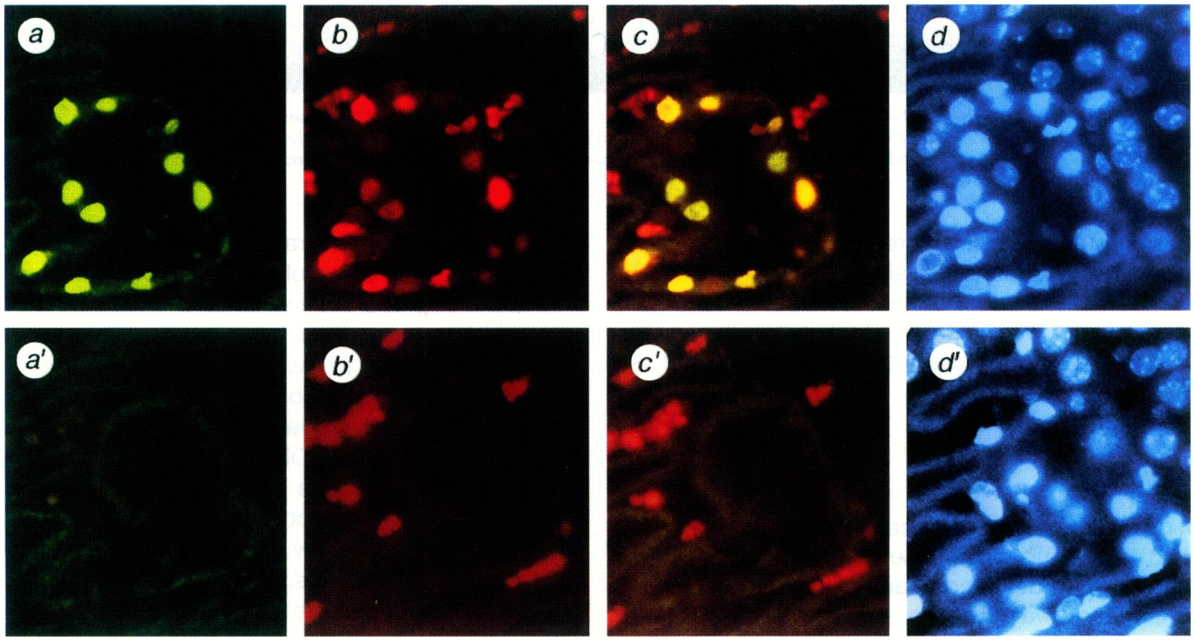

Figure 2. Ligase-dependent labeling of apoptotic cells in mouse kidney. **a** to **d** and **a′** to **d′** are consecutive sections of mouse kidney showing a tubule with several apoptotic cells close to the site of puncture of the capsule. The section shown in **a** to **d** was incubated successively with ligase/biotin-labeled A overhang hairpin probe, which was detected with fluorescein-avidin and then with terminal transferase/Texas Red-dUTP. The section in **a′** to **d′** was incubated with the same reagents but with the omission of both enzymes. **a** and **a′** were photographed in blue light to detect fluorescein, **b** and **b′** in yellow light to detect Texas Red, and **c** and **c′** in dual-wavelength light to detect both fluorochromes. **d** and **d′** were photographed with ultraviolet illumination to visualize DAPI staining of nuclei. The objects with red fluorescence visible in **b**, **b′**, **c**, and **c′** that do not stain with DAPI are red blood cells.

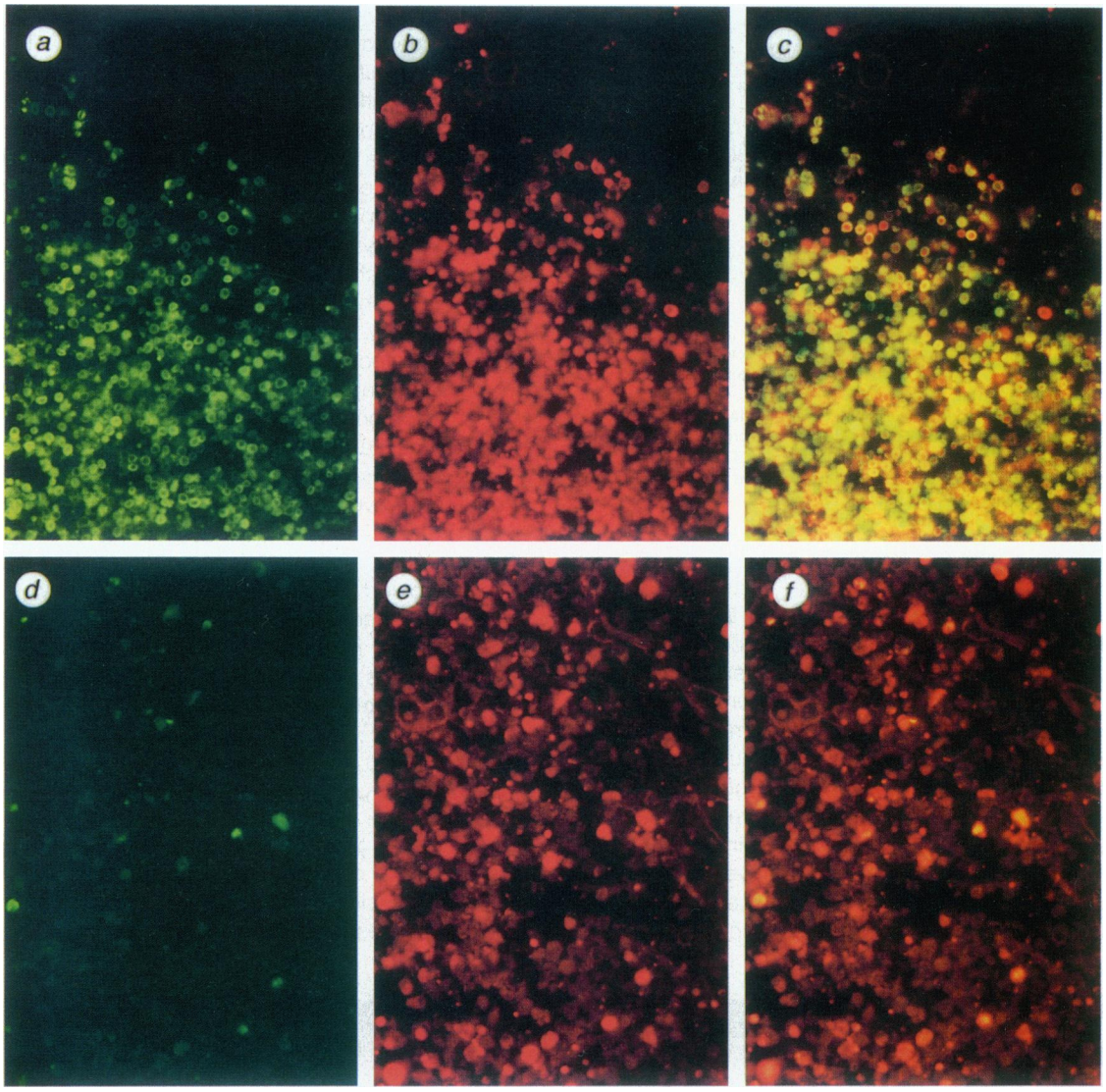

Figure 3. Comparison of the detection of DNA strand breaks by ligation of hairpin oligonucleotide probes and by terminal transferase labeling of 3′ ends. A section of dexamethasone-treated rat thymus (**a** to **c**) and a section of Wilms' tumor (**d** to **f**) were labeled by ligation of a single A overhang oligonucleotide hairpin probe (photographed by green fluorescence, **a** and **d**) and by terminal transferase (photographed by red fluorescence, **b** and **e**). The sections were also photographed with dual-wavelength light (**c** and **f**).

overhang probe produced a more intense signal but also showed more background (ie, signal in the absence of enzyme) than the other probes. Additionally, necrotic areas of the tumor were more intensely labeled by both the NN and blunt-ended probes than by the single A overhang.

Discussion

Hairpin oligonucleotide probes can be designed with any type of 3′ overhang complementary to double-strand DNA termini being detected. Placing the nonradioactive label in the loop away from the stem of the hairpin may avoid potential interference of the large biotin groups with the enzymatic linkage of the probe to the section by ligase.

The single-nucleotide A overhang probe appears to have the best specificity for labeling apoptotic cells. The production of double-strand breaks with 3′ overhangs in apoptotic cells may result from the action of an endonuclease with properties similar to DNAse I on chromatin DNA.[10] A major endonuclease involved in apoptosis is a caspase-activated DNAse.[18] Before the identification of this enzyme it was established that the apoptotic nuclease has properties similar to those of pancreatic DNAse I.[19] Specifically, 1) the DNA ends produced by DNAse I cleavage (5′-phosphate and 3′-hydroxyl) are the same as those found in apoptotic nuclei,[20–22] 2) DNAse-I-transfected COS cells show chromatin changes similar to those seen in apoptosis,[23] and 3) DNAse I cleavage of chromatin produces the same characteristic nucleosomal DNA fragments that can be isolated from apoptotic cells.[24] Although the apoptotic endonuclease produces random multiple single-strand nicks within internucleosomal DNA, these may be focused at the junction of the nucleosome and the internucleosomal region, because of

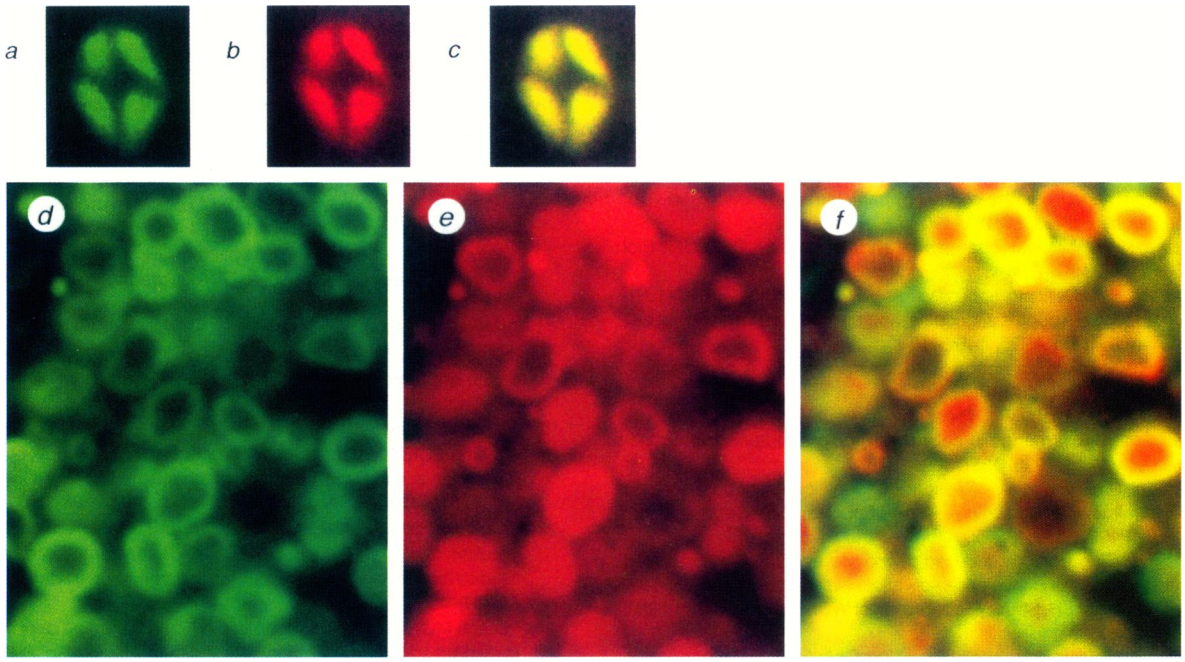

Figure 4. Confocal microscope images of apoptotic nuclei successively labeled by ligation of a single A overhang hairpin oligonucleotide probe (green, **a** and **d**) and by terminal transferase (red, **b** and **e**). The computer-generated combined green and red images are shown in **c** and **f**. **a** to **c**: A single apoptotic nucleus in mouse kidney. **d** to **f**: Dexamethasone-treated rat thymus.

the higher-order structure of chromatin in the nucleus.[25] The finding of single-base 3′ overhangs thus is consistent with limited access of chromatin to the apoptotic endonuclease, the staggered cleavage resulting from the helical twist of the DNA.[26] This analysis predicts that breaks with double-nucleotide overhangs may also be frequent. Better methods for the suppression of nonspecific binding of probes with double-nucleotide overhangs will be required to reliably detect breaks with overhangs of two and, presumably, greater numbers of nucleotides.

The methodology could be extended by the design of hairpins with 5′ overhangs to enable detection of 5′ overhang strand breaks. However, in this case, binding to single-stranded DNA fragments will occur because the recessed 3′ hydroxyl on the oligonucleotide would bind to 5′ phosphates on the section, which could be at the end of a single-stranded DNA fragment as well as a double-strand break with a 5′ overhang. Thus, the detection of 5′ overhangs would require dephosphorylation of the tissue section before ligation and the use of a 5′ phosphorylated oligonucleotide.

Hairpin oligonucleotide probes are equivalent in sensitivity and specificity to the double-stranded DNA probes made by the polymerase chain reaction (PCR) that we used previously.[10] However, problems of background (ligase-independent binding to the section) are reduced; some of the background when using PCR-derived probes is due to remnants of the PCR reaction, such as unincorporated nucleotides, which have not been purified away from the double-stranded product.

The ease of synthesis of hairpin oligonucleotide probes, their low cost, and the simple procedure for the detection of double-strand breaks, should enable this methodology to have wide application in staining of apoptotic cells, with potential extension to the detection of different kinds of double-strand breaks in dying cells, which may have mechanistic and diagnostic significance. However, it is important to recognize that the presence of double-strand breaks in nuclei is not conclusive that the cell is undergoing apoptosis. Breaks indistinguishable from those in apoptotic cells also occur in nuclei in very early necrosis.[17] Whereas apoptotic cells disappear rapidly after they are formed, necrotic cells are generally not subjected to a rapid removal from tissue.[8] After the initial phase in which double-strand breaks are present, there is a later very prolonged phase in which slow and random degeneration of the DNA yields many single-strand breaks that can be labeled by terminal transferase. The ligase-dependent attachment of hairpin oligonucleotides to tissue sections, described here, although having advantages over terminal transferase as a method for detection of apoptosis, must be accompanied by observations of morphological features of apoptosis, as has often been emphasized by those using the terminal transferase technique.

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